AF/1641 #IFW

PTO/SB/21 (09-04) MODIFIED

TRANSMITTAL FORM	**Application Number:**	10/028,741
	Filing Date:	December 20, 2001
	First Named Inventor:	Shinichiro Kurosawa
	Art Unit:	1641
	Examiner Name:	C. Kaufman
Total Number of Pages in this Submission : _____	**Attorney Docket Number:**	OMRF:004US

ENCLOSURES *(check all that apply)*

- ☐ Fee Transmittal Form
  - ☐ Fee Attached
- ☐ Amendment/Reply
  - ☐ After Final
  - ☐ Affidavits/declaration(s)
- ☐ Extension of Time Request
- ☐ Express Abandonment Request
- ☐ Information Disclosure Statement
  - ☐ Form PTO-1449
  - ☐ References _____
- ☐ Certified Copy of Priority Documents
- ☐ Reply to Missing Parts/ Incomplete Application
  - ☐ Reply to Missing Parts/Requirements
  - ☐ Declaration(s) _____
  - ☐ Copy of Notice of Missing Parts/Requirements
- ☐ Drawings(s) _____
- ☐ Licensing-related Papers
- ☐ Petition
- ☐ Petition to Convert to a Provisional Application
- ☐ Power of Attorney, Revocation, Change of Correspondence Address
  - ☐ Statement under 37 CFR §3.73(b)
  - ☐ Designation of Patent Practitioners
- ☐ Terminal Disclaimer
- ☐ Request for Refund
- ☐ CD, Number CD(s) _____
  - ☐ Landscape Table on CD
- ☐ After Allowance Communication to TC
- ☐ Appeal Communication to Board of Appeals and Interferences
- ☒ Appeal Communication to TC (Appeal Notice, Brief, Reply Brief)
- ☐ Proprietary Information
- ☐ Status Letter
- ☒ Other Enclosure(s) (please identify below)
  - ☒ Check in the amount of $250.00
  - ☒ Authorized to be charged to deposit account if check insufficient or inadvertently omitted Deposit account number: 50-1212/IOWA:046US/SLH
  - ☐ Sequence Statement
  - ☐ Paper Copy of Sequence Listing
  - ☐ Computer Readable Form (CRF)
  - ☒ Postcard

Remarks: If the check is inadvertently omitted or additional fees under 37 C.F.R. §§ 1.16 to 1.21 are required for any reason relating to the enclosed materials, the Commissioner is authorized to deduct said fees from Fulbright & Jaworski L.L.P. Account No.: 50-1212/IOWA:046USUS/SLH.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Fulbright & Jaworski, L.L.P.	Customer Number	32425
Signature			
Printed Name	Steven L. Highlander	Reg. No.	37,642
Date	August 12, 2005		

CERTIFICATE OF TRANSMISSION/MAILING

I hereby certify that this correspondence is being facsimile transmitted to the USPTO or deposited with the United States Postal Service with sufficient postage as first class mail in an envelope addressed to: MS APPEAL BRIEF - PATENTS; Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450 on the date shown below:

Signature			
Typed or Printed Name	Steven L. Highlander	Date	August 12, 2005

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shinichiro KUROSAWA and
Deborah J. Stearns-KUROSAWA

Serial No.: 10/028,741

Filed: December 20, 2001

For: METHOD FOR MONITORING COAGULABILITY AND HYPERCOAGULABLE STATES

Group Art Unit: 1641

Examiner: C. Kaufman

Atty. Dkt. No.: OMRF:004US

CERTIFICATE OF MAILING
37 C.F.R. 1.8

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: MS Appeal Brief – Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date below:

August 12, 2005
Date

Steven L. Highlander

BRIEF ON APPEAL

TABLE OF CONTENTS

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shinichiro KUROSAWA and
Deborah J. Stearns-KUROSAWA

Serial No.: 10/028,741

Filed: December 20, 2001

For: METHOD FOR MONITORING COAGULABILITY AND HYPERCOAGULABLE STATES

Group Art Unit: 1641

Examiner: C. Kaufman

Atty. Dkt. No.: OMRF:004US

BRIEF ON APPEAL

Mail Stop Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submits an original and two copies of this Appeal Brief. The deadline for filing the Brief is August 13, 2005, based on the receipt of appellants' Notice of Appeal by the United States Patent and Trademark Office on June 13, 2005. As August 13th is a Saturday, the actual deadline is August 15, 2005. The fee for this Appeal Brief is included herewith. No additional fees are believed due in connection with the instant paper. However, should appellants' check be missing, or any other fees be due, the Commissioner is authorized to withdraw the appropriate fee from Fulbright & Jaworski L.L.P. Deposit Account No. 50-1212/OMRF:004US/SLH.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

I. REAL PARTY IN INTEREST

The real party in interest is the assignee, the Oklahoma Medical Research Foundation, Oklahoma City, OK.

II. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

III. STATUS OF THE CLAIMS

Claims 1-30 were filed with the original application. Claims 17-30 were canceled in response to a restriction requirement. Claims 1-16 are pending, stand rejected, and are appealed. A copy of these claims is set forth in the attached Claims Appendix.

IV. STATUS OF AMENDMENTS

No amendments have been offered following the final Office Action.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention relates to methods for monitoring effective thrombin levels in patients undergoing anticoagulant therapy comprising measuring circulating levels of soluble endothelial protein C receptor (sEPCR), wherein lowered sEPCR levels relate to lowered effective thrombin activity. The anticoagulant therapy may involve a vitamin K antagonist, may involve at least one of Warfarin, Coumadine, Previscan, Sintrom, heparin, low molecular weight heparin, pentasaccharides, hirudin, hirudin analogs, coagulation factor inhibitors, protein C pathway components, tissue factor pathway inhibitors, anti-platelet compounds or fibrinolytic pathway components. The sEPCR may be measured by an immunoassay, for example, by ELISA. The circulating sEPCR levels may be determined from a sample such as a blood product (serum, plasma), cerebrospinal fluid or urine. **Specification at page 4, lines 10-20.**

In another embodiment, the present invention provides methods for monitoring the effectiveness of anticoagulant therapy comprising measuring circulating sEPCR levels, wherein decreases in sEPCR indicate that the anticoagulant therapy is effective. The anticoagulant therapy may involve a vitamin K antagonist, may involve at least one of Warfarin, Coumadine, Previscan, Sintrom, heparin, low molecular weight heparin, pentasaccharides, hirudin, hirudin analogs, coagulation factor inhibitors, protein C pathway components, tissue factor pathway inhibitors, anti-platelet compounds or fibrinolytic pathway components. The sEPCR may be measured by an immunoassay, for example, by ELISA. The circulating sEPCR levels may be determined from a sample such as a blood product (serum, plasma), cerebrospinal fluid or urine. **Specification at page 4, lines 21-30.**

VI. GROUND OF REJECTION TO BE REVIEWED ON APPEAL

Are claims 1-16 obvious under 35 U.S.C. §103 over Esmon *et al.* (Exhibit 1) and Kurosawa *et al.* (Exhibit 2) in further view of Hirsh *et al.* (Exhibit 3).

VII. ARGUMENT

As an initial matter, appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by "substantial evidence" within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *In re Gartside*, the Federal Circuit stated that "the 'substantial evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* At 1312. Accordingly, it necessarily follows that an Examiner's position

on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

A. Rejection Under 35 U.S.C. §103

Claims 1-16 are rejected under §103 as obvious over Esmon *et al.* (Exhibit 1) and Kurosawa *et al.* (Exhibit 2) in further view of Hirsh *et al.* (Exhibit 3). According to the examiner, Esmon *et al.* discloses that hirudin, a specific thrombin inhibitor, blocked thrombin mediated increases in circulating EPCR, and that circulating EPCR levels are thus a surrogate for thrombin levels. The examiner points to the following passage:

> This [monitoring of plasma EPCR] could prove useful in monitoring the progression of cardiovascular disease or the effectiveness of therapeutic interventions in these [human] patients.

Esmon *et al.* at page 255. From this, it is concluded that the reference adequately discloses the use of sEPCR assays to monitor the effectiveness of anticoagulant therapies. Kurosawa *et al.* is cited as teaching the detection of sEPCR by ELISA of patient plasma. Hirsh is cited for teaching various anticoagulant therapies, and measuring the effectiveness of the anticoagulant therapy.

B. The References do not Reasonably Suggest Success in Humans

As discussed previous responses, the cited passages from Esmon *et al.* refer to studies by Gu *et al.* (2000) (Exhibit 4) in which endotoxin or thrombin treatment elevated soluble EPCR levels in a rat experimental model. These are ***inflammatory*** mediators in sepsis, and the effect of thrombin was blocked by hirudin, a specific thrombin inhibitor. By way of discussing their results, both Gu *et al.* and Esmon *et al.* extrapolated these observations in a rat model to the human condition by stating that monitoring plasma EPCR levels ***may*** indicate thrombin-

mediated large vessel disease activity, and ***might*** prove useful to monitor disease progression or effectiveness of therapeutic interventions in these patients.

However, the study described in Gu *et al.* (2000) was designed to investigate the mechanism by which soluble EPCR is generated using an *in vivo* model. It was not designed to investigate how soluble EPCR levels change in patients as a result of ***anticoagulant therapy***. Their data predicts that soluble EPCR levels ***may*** be linked to thrombin production in humans, but they do not test the prediction. They most certainly do not show data from patients or other humans in their study.

The examiner attempts to counter this by citing again from Esmon *et al.* at page 255, column 2, that "soluble EPCR ... can increase severalfold in autoimmune disorders or in septic shock." Does that statement suggest, much less provide any credible evidence, that anticoagulant therapy in humans can be monitored using the claimed methods? Appellants submit that it does not. Thus, we are still left with an unbridged gap between animal studies and humans.

Clearly, the need to conduct studies in humans is at the crux of the rejection, namely, the lack of likelihood of success in practicing the claimed invention based only animal studies. *In re Vaeck*, 20 USPQ2d 1438 (Fed. Cir. 1991) (holding that a reasonable likelihood of success in practicing the invention is required for any *prima facie* case of obviousness). The rejection here is based on the assertion that observations on sEPCR levels in a rat experimental model are predictive of levels in a corollary human condition. The scientific literature contains examples in which such extrapolations hold, and others in which they do ***not***. There is an inherent limitation for data collected in an animal model, namely, that such data are clearly representative of the

animal model, but may not be representative of similar response in humans (or even other animal models for that matter).

Despite protesting, the examiner has indeed simply dismissed this line of argumentation as "not persuasive," claiming that sufficient evidence exists to permit this extrapolation. The examiner repeatedly points to the previously quoted portions of Esmon *et al.*, and further notes that mutations in both human and murine thrombin genes can lead to thrombosis. However, the binding of thrombin to its receptor, which initiates signaling and the production of sEPCR, is ***distinct*** from its clotting function, which is an acellular phenomenon. Thus, this observation is not particularly relevant to the present rejection.

Giudici *et al.* (1999) (Exhibit 5) was cited as teaching that both humans and mice can be protected from sepsis by antithrombin. In point of fact, Giudici *et al.* did ***not*** report that humans can be protected from sepsis by antithrombin, with the results of the trial being very disappointing. All that this paper demonstrates is that antithrombin therapy ***may*** be effective in only a very small sub-population of patients with sepsis. This sub-population is very closely defined as patients with all of (a) sepsis, (b) septic shock, (c) requiring hemodynamic support and (d) antithrombin levels <70% of normal at the time of admission. This is ***not*** merely "patients with sepsis." Furthermore, there was ***no*** difference between placebo or antithrombin treatment on overall mortality beyond 30 days. From a clinical perspective, the patient sub-population affected was so narrowly defined as to be useless on a practical basis. As a result, antithrombin therapy is not recommended for use in sepsis.

Nonetheless, the examiner concludes from this study that though "animal models are not ... exact replicas for human responses, ... [they] have and continue to serve as valuable models for many human diseases/conditions." Indeed, rodents do provide valuable information

regarding human disease states. That is not the issue here. The question is whether one can make a sufficient extrapolation from the animal studies of Esmon *et al.* such that there would be ***reasonable*** predictability in a comparable human system to render the present claims obvious. In order to more fully understand the issue, appellants wish to provide a more detailed discussion of the invention and it's development.

As discussed previously, the invention relates to measuring soluble EPCR in humans. The inventors are the first to show that soluble EPCR levels are related to thrombin levels. The soluble EPCR seen in the human assay is released from the membrane-bound parent by metalloproteinase activity. The metalloproteinase activity is up-regulated through a series of cellular signaling events arising from thrombin's interaction with cells that express EPCR. These receptors are called protease-activated receptors (PARs), of which several have now been identified.

The important point to be made about PARs is that humans utilize PAR-1 and PAR-4. Kahn *et al.* (1999; Exhbit 6). In contrast, mice do not have PAR-1, and use PAR-3 and PAR-4 instead (Kahn *et al.* (1998); Exhibit 7).[1] Thus, the inventors were prompted to perform the experiments described in the instant patent application because the they were aware that thrombin receptors differed between mice and humans (not to mention other species) and the signaling pathways are, therefore, different as well. See, for example, Connolly *et al.* (1994; Exhibit 8). Thus, the results of Esmon *et al.*, though interesting, did not provide *a priori* predictability with regard to sEPCR levels and thrombin activity in humans. Appellants submit that because mice and humans (not to mention other species) have different PAR receptors, there was no reason to believe that what Esmon *et al.* showed was true for thrombin-thrombin receptor

[1] With regard to PAR-2, the distinction between species is not clear, and PAR-2 is used by many enzymes other than thrombin.

interactions in a mice would predictably translate to humans. This unrebutted fact is far more relevant than the examiner's citations.

In the Advisory Action, the examiner attempts to sweep these important facts away by stating that "though there are different homologous receptors used in humans and mice, there is no expectation that the *intracellular* events are not at least on whole the same in rodents and humans." ***Where is the evidence supporting this statement?*** This appears nothing more than conjecture on the part of the examiner, and thus it does not rise to a level necessary for a showing sufficient to support obviousness. Further, the attempt to circumvent this argument by once again pointing to the Kurosawa *et al.* paper is not availing ***since that paper did not deal with therapy!***

Moreover, the examiner has improperly dismissed the impact of genetic variability on the results seen here. Murine populations used for studies like those described by Esmon *et al.* are inbred. While that may not have a significant impact in certain situations (for example, orthotopic cancer models), it most certainly has an impact when dealing with the complicated response seen with regard to sepsis and clotting. Even where positive results are seen in mice (or rats), there would clearly be a need to confirm the results in human, and the literature is replete with examples where the murine results could ***not*** be confirmed in humans (which the examiner readily admits). In response, the examiner simply concludes, without ***any*** basis, that such variability (or other reasons for distinguishing murine studies from humans) does not preclude predictability. This statement is factually incorrect, and in any event, unsupported from a legal standpoint.

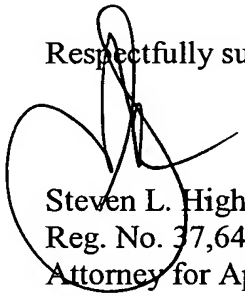
C. Summary

In sum, appellants submit that a fair review of the record indicates that the examiner arguments in favor of extrapolating from mice to humans in the context of thrombin and sEPCR are far less compelling than appellants' arguments to the contrary. On this basis, it is submitted that there was an insufficient likelihood of success in practicing the claimed invention, and therefore no *prima facie* case of obviousness exists. Reversal of the rejection based on the preceding discussion is respectfully requested.

VIII. CONCLUSION

It is respectfully submitted, in light of the above, that none of the pending claims are properly rejected under 35 U.S.C. §103. Reversal of the pending grounds for rejection is thus respectfully requested.

Respectfully submitted,



Steven L. Highlander
Reg. No. 37,642
Attorney for Appellants

FULBRIGHT & JAWORSKI L.L.P
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 536-3184

Date: August 12, 2005

CLAIMS APPENDIX

1. A method for monitoring effective thrombin levels in a human patient undergoing anticoagulant therapy comprising measuring circulating levels of soluble endothelial protein C receptor (sEPCR) of said patient, wherein lowered sEPCR levels relate to lowered effective thrombin activity.

2. The method of claim 1, wherein the anticoagulant therapy involves a vitamin K antagonist.

3. The method of claim 1, wherein the anticoagulant therapy involves at least one of Warfarin, Coumadine, Previscan, and Sintrom.

4. The method of claim 1, wherein the anticoagulant therapy involves use of heparin, low molecular weight heparin, pentasaccharides, hirudin, hirudin analogs, coagulation factor inhibitors, protein C pathway components, tissue factor pathway inhibitors, anti-platelet compounds or fibrinolytic pathway components.

5. The method of claim 1, wherein the sEPCR is measured by an immunoassay.

6. The method of claim 5, wherein the sEPCR is measured by ELISA.

7. The method of claim 1, wherein the sEPCR level is determined by measuring sEPCR in a blood product, cerebrospinal fluid or urine.

8. The method of claim 7, wherein the blood product is plasma or serum.

9. A method for monitoring effectiveness of anticoagulant therapy in a human patient comprising measuring circulating sEPCR levels of said patient, wherein decreases in sEPCR indicate that the anticoagulant therapy is effective.

10. The method of claim 9, wherein the anticoagulant therapy involves a vitamin K antagonist.

11. The method of claim 9, wherein the anticoagulant therapy involves at least one of Warfarin, Coumadine, Previscan, and Sintrom.

12. The method of claim 9, wherein the anticoagulant therapy involves use of heparin, low molecular weight heparin, pentasaccharides, hirudin, hirudin analogs, coagulation factor inhibitors, protein C pathway components, tissue factor pathway inhibitors, anti-platelet compounds or fibrinolytic pathway components.

13. The method of claim 9, wherein the sEPCR is measured by an immunoassay.

14. The method of claim 13, wherein the sEPCR is measured by ELISA.

15. The method of claim 9, wherein the sEPCR level is determined by measuring sEPCR in a blood product, cerebrospinal fluid or urine.

16. The method of claim 15, wherein the blood product is plasma or serum.

EVIDENCE APPENDIX

Exhibit 1: Esmon *et al.*

Exhibit 2: Kurosawa *et al.*

Exhibit 3: Hirsh *et al.*

Exhibit 4: Gu *et al.*

Exhibit 5: Giudici *et al*

Exhibit 6: Kahn *et al.* (1999)

Exhibit 7: Kahn *et al.* (1998)

Exhibit 8: Connolly *et al.*

Identification of Functional Endothelial Protein C Receptor in Human Plasma

Shinichiro Kurosawa,* Deborah J. Stearns-Kurosawa,* Noriko Hidari,* and Charles T. Esmon*‡§

*Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104; ‡Department of Pathology and Department of Biochemistry and Molecular Biology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104; and §Howard Hughes Medical Institute, Oklahoma City, Oklahoma 73104

Abstract

The endothelial cell protein C receptor (EPCR) binds protein C and facilitates activation by the thrombin–thrombomodulin complex. EPCR also binds activated protein C (APC) and inhibits APC anticoagulant activity. In this study, we detected a soluble form of EPCR in normal human plasma. Plasma EPCR appears to be ~ 43,000 D, and circulates at ~ 100 ng/ml (98.4±27.8 ng/ml, n = 22). Plasma EPCR was purified from human citrated plasma using ion exchange, immunoaffinity, and protein C affinity chromatography. Flow cytometry experiments demonstrated that plasma EPCR bound APC with an affinity similar to that previously determined for recombinant soluble EPCR ($K_{d_{app}}$ = 30 nM). Furthermore, plasma EPCR inhibited both protein C activation on an endothelial cell line and APC anticoagulant activity in a one-stage Factor Xa clotting assay. The physiological function of plasma EPCR is uncertain, but if the local concentrations are sufficiently high, particularly in disease states, the present data suggest that the soluble plasma EPCR could attenuate the membrane-bound EPCR augmentation of protein C activation and the anticoagulant function of APC. (*J. Clin. Invest.* 1997. 100:411–418.) Key words: soluble protein C receptor • activated protein C • protein C • anticoagulation • endothelial cells

Introduction

The clinical importance of the protein C pathway is evidenced by the multitude of dysfunctions in this pathway that result in thrombosis (1, 2). Activation of protein C to its active serine protease, activated protein C (APC),[1] initiates a series of events that play a key role in the regulation of blood coagulation. Protein C and APC have also been implicated in the regulation of the host response to inflammation. Patients with meningococcemia (3) or streptococcal sepsis (4) with purpura fulminans and acquired protein C deficiency have been treated with protein C concentrate with a favorable clinical outcome. Furthermore, the response in primates to low-level bacterial infusion intensified when the protein C pathway was blocked (5). APC also protected primates and rodents from lethal levels of *Escherichia coli* (5, 6).

Endothelial cells play a critical role in the protein C pathway in that they express two of the known receptors responsible for efficient protein C activation: thrombomodulin, and the endothelial protein C/APC receptor (EPCR) (7, 8). Thrombomodulin (CD141) is a transmembrane cofactor that binds circulating thrombin with high affinity, and the resultant enzyme–cofactor complex is required for physiologically relevant protein C activation rates (9, 10). EPCR is a recently identified receptor with significant homology to the CD1/MHC class 1 family (7, 11, 12). EPCR binds both protein C and APC with similar affinity ($K_{d_{app}}$ ~ 30 nM) (11), and facilitates protein C activation by presenting the protein C substrate to the thrombin–thrombomodulin activation complex (8). Both endothelial cell receptors are type 1 transmembrane protein in which the ligand binds to an extracellular domain, and both have a short intracellular cytoplasmic tail (11, 13–15). In addition, their in vitro cell surface expression is downregulated similarly by TNF-α (7).

The characteristics of soluble forms of the receptors, however, differ in several respects. Recombinant soluble thrombomodulin has reduced cofactor activity relative to the membrane form (16, 17). With both purified components and with cells, the changes in thrombin's substrate specificity induced by thrombomodulin result from competition for a shared binding domain on thrombin as well as conformational alterations in the active site pocket (18–24). Soluble thrombomodulin also accelerates inactivation of thrombin by a variety of inhibitors (25, 26). Both plasma and urine contain detectable thrombomodulin (27, 28), and because the thrombomodulin gene does not contain introns (13), these soluble forms are due to proteolysis of the extracellular domain at the cell surface. Soluble degradation products of thrombomodulin in plasma are known markers of endothelial cell damage in a variety of disease states (27, 29–34), and are comprised of a mixture of thrombin-binding fragments with varying reduced affinities, as well as nonbinding fragments (27).

In contrast, recombinant soluble EPCR (rsEPCR), truncated just before the transmembrane domain, binds both protein C and APC with an affinity similar to that observed for intact cell surface–expressed EPCR (11). APC anticoagulant activity is inhibited effectively when bound to rsEPCR (12), presumably because both rsEPCR and Factor Va share binding determinants in a groove reminiscent of the anion-binding exosite I in thrombin occupied by thrombomodulin (35). rsEPCR, however, appears to influence neither proteolysis of small synthetic substrates by APC, nor inactivation of APC by α1-antitrypsin or protein C inhibitor (12). Unlike membrane-

Address correspondence to Charles T. Esmon, Ph.D., 825 N.E. 13th Street, Oklahoma City, OK 73104. Phone: 405-271-6474; FAX: 405-271-3137; E-mail: charles-esmon@omrf.uokhsc.edu

Received for publication 31 January 1997 and accepted in revised form 18 April 1997.

1. *Abbreviations used in this paper:* APC, activated protein C; EPCR, endothelial protein C receptor; fl-APC, fluorescein in the active site; rsEPCR, recombinant soluble EPCR with the HPC4 epitope inserted in place of the transmembrane domain and cytosolic tail.

J. Clin. Invest.

0021-9738/97/07/0411/08 $2.00
Volume 100, Number 2, July 1997, 411–418

bound EPCR which enhances protein C activation (8), rsEPCR has little effect on protein C activation by the soluble thrombin–thrombomodulin complex (12), suggesting that any soluble forms of EPCR might inhibit protein C activation by competing with membrane-associated EPCR for protein C.

These observations and considerations led to the current studies investigating possible plasma forms of EPCR and their potential impact on the protein C pathway. We identify for the first time plasma EPCR, and show that purified plasma EPCR blocks cellular protein C activation and APC anticoagulant activity.

Methods

Materials. The following reagents were purchased from the indicated vendors: porcine intestinal mucosal heparin, diisopropyl fluorophosphate, biotinamidocaproate *N*-hydroxysuccinimide ester, and bovine serum albumin (Sigma Chemical Co., St. Louis, MO); Spectrozyme PCa (American Diagnostica, Inc., Greenwich, CT); ELISA amplification kit (GIBCO BRL, Gaithersburg, MD); AffiGel-10 (Bio-Rad Laboratories, Hercules, CA); Hanks' balanced salt solution and 3-(*N*-morpholine)propane sulfonic acid (Mops) (Fisher Scientific Co., Fairlawn, NJ). All other reagents were of the highest quality commercially available.

Proteins. Human protein C (36), bovine thrombin (37), and bovine antithrombin (38) were purified as described. rsEPCR consists of the extracellular domain of EPCR truncated at residue 210 just before the transmembrane domain, followed by a 12-residue sequence that permits calcium-dependent immunoaffinity purification on the HPC4 monoclonal antibody (36, 39). The construction, purification, and protein C/APC–binding characteristics of rsEPCR have been described previously (11). Goat preimmune serum and polyclonal antiserum to rsEPCR was prepared, and the IgG was purified as previously described (11). Goat anti–rsEPCR polyclonal antibody was biotinylated with biotinamidocaproate *N*-hydroxysuccinimide ester using standard procedures.

Monoclonal antibodies. mAbs against rsEPCR were obtained as described for other proteins (36). The 1494, 1495, and 1496 mAbs are IgG1 κ antibodies that bind to rsEPCR and to cell surface–expressed EPCR. The 1494 and 1496 mAbs block the binding of protein C and APC to EPCR, and inhibit the ability of cellular EPCR to facilitate protein C activation by the thrombin–thrombomodulin complex (8). The 1495 mAb does not block ligand binding to EPCR, does not alter cell surface protein C activation, and has a binding epitope distinct from that for 1494 or 1496 mAb (data not shown). The 1494 mAb was biotinylated with biotinamidocaproate *N*-hydroxysuccinimide ester using standard procedures. The 1496 mAb was coupled to AffiGel-10 according to the manufacturer's directions for immunoaffinity purification of plasma EPCR. The screening of anti-EPCR mAb was done using methods previously reported (8, 11).

Clotting assay. The effect of rsEPCR or purified plasma EPCR on APC (25 nM) anticoagulant activity in a one-stage Factor Xa clotting assay was performed as described (12) in the presence or absence of 83 μg/ml 1496 mAb, an antibody that blocks APC–EPCR interaction (8). The soluble EPCRs and 1496 mAb were preincubated for 15 min before assay.

Cell culture. All human cell lines were maintained as described previously (11). EA.hy926 cells, a transformed human endothelial cell line (40), were kindly provided by Cora-Jean Edgell (University of North Carolina at Chapel Hill).

Flow cytometric analysis. To serve as a fluorescent probe, APC was labeled with fluorescein in the active site (fl-APC) as described (7, 41). The effect of rsEPCR or plasma EPCR on APC binding to EA.hy926 cells was studied by flow cytometry using methods described previously (11). In brief, harvested cells were incubated for 30 min on ice with 60 nM fl-APC in the absence or presence of increasing concentrations of either soluble EPCR preparation, were washed, and cell-bound fluorescence was determined by flow cytometry with 10,000 events counted per sample. All assays were done in Hanks' balanced salt solution supplemented with 1% BSA, 3 mM $CaCl_2$, 0.6 mM $MgCl_2$, and 0.02% sodium azide.

Cell surface protein C activation. EA.hy926 cells were cultured in 96-well tissue culture dishes as described (8). The confluent monolayers were washed three times with Hanks' balanced salt solution supplemented with 1% (wt/vol) BSA, 3 mM $CaCl_2$, 0.6 mM $MgCl_2$, and 0.02% sodium azide. All assays were done at room temperature in the same buffer in 60 μl final volume, and all protein concentrations represent the final concentration in the assay. Protein C was added (0.1 μM) in the absence or presence of rsEPCR, plasma EPCR, or 1494 mAb at the indicated concentrations, and was preincubated with the cells for 15 min. Thrombin was added to the mixtures (2 nM) to start the activation reactions. At the indicated time, 50 μl aliquots were removed and added to 10 μl of antithrombin (0.7 μM final) and heparin (5 U/ml final) in a 96-well microtiter plate. APC amidolytic activity was determined by addition of Spectrozyme PCa substrate (0.2 mM) and the rate of change in absorbance at 405 nm (mOD/min) was measured (V_{max} kinetic microplate reader; Molecular Devices, Menlo Park, CA). All assay points were done in triplicate wells, and < 10% of the protein C substrate was activated as determined by reference to a standard curve of fully APC versus mOD/min.

Plasma and serum collection. Whole blood was collected from normal adult volunteers (12 females and 10 males) by venipuncture into 3.8% buffered citrate solution, or into tubes without anticoagulant (Vacutainer tubes; Becton Dickinson, Franklin Lakes, NJ). No screening of donors was attempted with respect to age, diet, or other variables. All volunteers were informed of the study and gave their written consent. The blood was centrifuged at 1,160 *g* for 10 min. The plasma and serum were aliquoted and stored frozen at −80°C until assay.

ELISA for quantitation of plasma EPCR. An ELISA for detection of EPCR antigen in plasma was developed. Microtiter plates (Maxisorp; NUNC NS, Roskilde, Denmark) were coated with 50 μl of 4 μg/ml 1495 mAb in 15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6, at 4°C overnight. The following steps were done at room temperature. The wells were washed three times with 20 mM Tris-HCl, 0.1 M NaCl, 0.05% Tween 20, pH 7.5 (assay buffer), and were blocked with assay buffer containing 0.1% (wt/vol) gelatin for at least 1 h. The wells were washed, 50-μl samples were added in triplicate wells, and the plates were incubated for 1 h. The wells were aspirated, washed three times with assay buffer, and 50 μl of 2 μg/ml biotin–1494 mAb was added. The plates were incubated for 1 h, washed three times, and 50 μl of 0.25 μg/ml streptavidin–alkaline phosphatase conjugate (GIBCO BRL) was added and incubated for one additional hour. The wells were washed five times, and the substrate and amplifier reagents from an ELISA amplification kit (GIBCO BRL) were added sequentially at 15-min intervals according to the manufacturer's directions. The color development was stopped with 0.3 M H_2SO_4, and the endpoint absorbance at 490 nm was read on a V_{max} microplate reader. Each plate contained standards in triplicate wells from 1.5–100 ng/ml rsEPCR in 20 mM Tris-HCl, 0.1 M NaCl, and 1 mM EDTA, 0.1% gelatin, pH 7.5. The standard curve was linear (r = 0.99) from 1.5–12.5 ng/ml, and plasma samples were diluted with the same buffer to fall within the linear range. Preliminary experiments determined that a final concentration of 1–2% human plasma did not affect the linearity or sensitivity of the standard curve. Plasma samples from healthy volunteers were diluted with assay buffer containing 1 mM EDTA to a final 2% plasma, and EPCR antigen levels were calculated from the average of triplicate wells by reference to a standard curve determined on the same plate.

Western blot. SDS-PAGE of plasma or serum samples was done with 10% acrylamide gels with the Laemmli buffer system (42) under nonreducing conditions using standard procedures. Gels were transferred to polyvinylidine membranes (PVDF; Millipore Corp., Bed-

ford, MA), the membranes were blocked, and were then incubated for 30 min with either preimmune goat IgG (50 μg/ml), or with a goat anti-rsEPCR polyclonal IgG (50 μg/ml). After washing, membranes were incubated with mouse anti-goat IgG-horseradish peroxidase conjugate (Pierce, Rockford, IL) at a 1:20,000 dilution for 30 min. Membranes were washed, and bound antibody-enzyme conjugate was detected with an enhanced chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Immunoadsorption. Serum or citrated plasma samples (400 μl) from healthy volunteers were incubated with 50 μl of 1495 mAb conjugated to AffiGel-10 (5 mg IgG/ml resin) overnight at 4°C with mixing. The samples were centrifuged, the supernatant was removed, and the resin was washed three times with 1 ml of 20 mM Tris-HCl, 0.1 M NaCl, and 0.02% sodium azide, pH 7.5. SDS-PAGE sample buffer containing a final 20 mM dithiothreitol was added to the washed resin, and the samples were boiled for 3 min, and processed for SDS-PAGE and Western blotting. Membranes were probed with biotinylated goat anti-rsEPCR polyclonal antibody at 4 μg/ml, and bound antibody was detected with a streptavidin-horseradish peroxidase conjugate (Pierce) and enhanced chemiluminescence detection system. Preliminary experiments determined that preadsorption of samples with 100 μl of Tris-inactivated AffiGel-10 resin for 1–4 h at room temperature followed by overnight immunoadsorption with the 1495 mAb AffiGel-10 gave identical Western blotting results (data not shown).

Purification of plasma EPCR. Plasma EPCR was purified from human citrated plasma (Oklahoma Blood Institute, Oklahoma City, OK) using a combination of ion-exchange chromatography, anti-rsEPCR mAb immunoaffinity chromatography, and chromatography on protein C affinity columns. Two preparations were done in slightly different ways.

In the first preparation, plasma (1 liter) was diluted with an equal volume of 20 mM Tris-HCl, pH 7.5, 10 mM benzamidine, 400 U sodium heparin and batch-adsorbed for 1 h with 1 g preswollen QAE resin. After settling, the resin was processed for purification of protein C as previously described (36). Solid ammonium sulfate was added to the supernatant at 4°C to 40% saturation, was centrifuged, and additional ammonium sulfate was added to that supernatant to achieve 70% saturation. After centrifugation, the soft pellet was placed in dialysis bags and dialyzed overnight against 12 liters of 20 mM Tris-HCl, 0.02% sodium azide, pH 7.4. The dialysate was applied to a 1496 mAb AffiGel-10 immunoaffinity column (6 ml resin; 5 mg IgG/ml resin) equilibrated in 20 mM Tris-HCl, 0.1 M NaCl, 0.02% sodium azide, pH 7.4. The column was washed with > 12 ml of the same buffer, and was eluted with 50% (vol/vol) ethylene glycol in 20 mM Tris-HCl, pH 7.4 (Xu, J., unpublished observations). The peak fractions from the elution were pooled (0.37 total OD_{280}), concentrated (Centriprep 30; Amicon Inc., Beverly, MA), and the buffer was changed to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.02% sodium azide, pH 7.4. This material was applied to a protein C affinity column that had been prepared previously by applying the purified protein C (3 mg) to an HPC4 AffiGel-10 column (5 mg IgG/ml resin; 0.9 × 8 cm) in the same buffer (see Fig. 3 *A* for chromatogram). The HPC4 mAb binds the protein C activation region in a calcium-dependent fashion (36, 39), and does not interfere with subsequent binding of EPCR to protein C. After applying the sample containing plasma EPCR, the column was washed with ~ 12 ml of buffer, and was eluted with 20 mM Tris-HCl, 0.1 M NaCl, 5 mM EDTA, 10 mM Mops, 0.02% sodium azide, pH 7.5. Fractions were monitored for absorbance at 280 nm, and for EPCR antigen using the ELISA described above. The eluate containing both protein C and plasma EPCR was applied to an FPLC Mono Q column (Pharmacia Fine Chemicals, Uppsala, Sweden), and the column was developed with a linear gradient of 0.1–1 M NaCl in 20 mM Tris-HCl, pH 7.5. About half of the plasma EPCR did not bind to the Mono Q column, half eluted at ~ 0.2 M NaCl, and protein C eluted at ~ 0.5 M NaCl. Both ionic species of plasma EPCR appeared identical on SDS-PAGE gels under reducing or nonreducing conditions with silver staining, with Coomassie BB staining, or with gold staining (Pierce) after transfer to PVDF membranes, and on Western blots with the biotin polyclonal anti-rsEPCR antibody probe (data not shown).

The second preparation of plasma EPCR was done with 4 liters of plasma to purify enough protein for functional studies. In this case, the 1496 AffiGel-10 resin (20 ml of 5 mg IgG/ml resin) was added directly to the citrated plasma, along with final concentrations of 10 mM benzamidine, 1 mM diisopropylfluorophosphate, and 0.5 U/ml sodium heparin. The plasma was batch-adsorbed overnight at 4°C with gentle mixing. After the resin settled, the supernatant was processed for protein C purification as described (36). The resin was packed into a 2.5 × 30 cm column, washed extensively with 20 mM Tris-HCl, 0.1 M NaCl, 0.02% sodium azide, pH 7.4, and was eluted with 50% ethylene glycol in 20 mM Tris-HCl, pH 7.4. The eluate was pooled and concentrations (5.5 total OD_{280}), was applied to a Mono Q column, and the two ionic species (breakthrough and 0.2 M NaCl eluate peak) were reapplied to the 1496 AffiGel-10 resin (1.5 × 11 cm). The column was eluted with 50% ethylene glycol as before. The eluate (0.71 ODs) was concentrated, and the buffer was changed to 20 mM Tris-HCl, 0.1 M NaCl, 3 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.02% sodium azide with a Centriprep 30. This material was then applied to an affinity column in which protein C (2.9 mg) had been applied initially in the same buffer to an HPC2 AffiGel-10 column (0.6 × 17 cm). The HPC2 mAb binds to the protein C serine protease domain, and does not interfere with EPCR binding (11). The bound EPCR was eluted with buffer containing 5 mM EDTA. Contaminating serum amyloid P (from the protein C sample) was removed by ion-exchange chromatography on the FPLC Mono Q column. The sample was applied in 0.2 M NaCl, so that the plasma EPCR did not bind, and was separated from the contaminants that eluted at 0.4–0.5 M NaCl. The resultant purified plasma EPCR (0.193 OD_{280}) appeared homogenous by SDS-PAGE with silver staining and by Western blotting with polyclonal anti-rsEPCR. This material was then used for the functional studies and amino-terminal sequence analysis.

Protein sequencing. The amino-terminal sequence analysis of soluble plasma EPCR was performed in Dr. Kenneth Jackson's laboratory (Molecular Biology Research Facility, W.K. Warren Medical Research Institute, Oklahoma City). Amino acids are designated by the standard one-letter code.

Results

Previous investigations into the function of EPCR found that protein C binding to the membrane form of EPCR resulted in facilitation of protein C activation by the thrombin–thrombomodulin complex on cell surfaces (8), but that soluble recombinant EPCR inhibited APC anticoagulant activity (12). These observations, along with the knowledge that soluble thrombomodulin degradation products in plasma are a marker of endothelial damage in various disease states (27, 29–34), led to the question of whether a soluble circulating form(s) of EPCR existed, and, if so, what role it may have in the protein C pathway.

As a first approach, plasma and serum samples from three healthy volunteers were diluted (4% vol/vol), run on 10% SDS-PAGE gels under nonreducing conditions, and processed for Western blotting using a goat polyclonal antibody raised against rsEPCR. As shown in Fig. 1 *A*, a single band of ~ 43,000 D appears in both the serum (lanes *2–4*) and plasma samples (lanes *5–7*) after the membrane is probed with the polyclonal antibody. The size of the protein detected appears slightly larger than the rsEPCR (Fig. 1 *A*, lane *1*). The other bands detected were background binding of IgG as judged by probing with preimmune IgG and longer exposure times (data not shown). Overnight incubation of plasma samples with the anti-EPCR 1495 mAb coupled to AffiGel-10 resin, followed by washing and elution of bound antigen under reducing condi-

tions, resulted in a single band detected by Western blotting with biotinylated goat anti-rsEPCR polyclonal antibody (Fig. 1 *A*, lanes *8* and *9*).

The observation that EPCR antigen was detectable directly from healthy donor plasma and serum, and appeared to be a single size, was surprising based on previous observations with thrombomodulin. Multiple soluble proteolytic products of thrombomodulin have been demonstrated (27, 28), and normal levels of these soluble products are quite low in the circulation, on the order of 10–40 ng/ml (27, 43). Determination of soluble EPCR antigen in plasma from healthy volunteers by ELISA found antigen levels of 91.1±24.5 ng/ml in females (n = 12) and 107.2±30.2 ng/ml in males (n = 10). When calculated together, the average plasma EPCR antigen level was 98.4± 27.8 ng/ml. The value for males appeared to be slightly higher than for females, similar to thrombomodulin (43), but the population studied was too limited for statistical analysis, and this study was not designed to assess differences due to gender, age, diet, or other variables.

Since the plasma EPCR appeared to be a single species at ~ 100 ng/ml, it became important to determine whether the circulating EPCR could bind protein C and APC. Soluble EPCR was purified from human plasma by a combination of ion-exchange chromatography, precipitation with ammonium sulfate, and immunoadsorption by anti-EPCR 1496 mAb-AffiGel-10 column chromatography as described in Methods.

This plasma EPCR (~ 110 μg) was applied to a protein C affinity column prepared by applying protein C (3 mg) to an antiprotein C HPC4 mAb AffiGel-10 column in buffer containing calcium and magnesium (Fig. 2 *A*). The plasma EPCR bound to the protein C affinity column, and was eluted with buffer containing EDTA. More than 98% of the applied plasma EPCR antigen bound to the protein C affinity column. The absorbance profile indicates coelution of EPCR and protein C from the antibody column, consistent with the calcium dependence of protein C binding to this antibody (39).

To purify sufficient protein for functional and structural studies, EPCR was purified from 4 liters of plasma using a similar, but slightly modified procedure, as described in Methods. After elution from a protein C antibody affinity column, residual contaminating proteins were removed by ion-exchange chromatography on an FPLC Mono Q column. The resultant preparation of plasma EPCR appeared homogenous on SDS-PAGE 10% gels with silver staining (Fig. 1 *B*, lanes *1* and *2*) and identical results were obtained with Western blots probed with biotin goat anti–rsEPCR polyclonal antibody under both reducing and nonreducing conditions (Fig. 1 *B*, lanes *3* and *4*). Amino-terminal sequence analysis of the purified protein yielded only one sequence, S-Q-D-A-S-D, which is identical to the amino-terminal sequence of rsEPCR (11). This is the first amino-terminal sequence determination of EPCR from a natural source.

The ability of plasma EPCR to bind to APC was assessed by competition studies in which plasma EPCR was allowed to compete with cellular EPCR for APC, and the resultant free APC that could bind to cellular EPCR was assessed by flow cytometry (Fig. 2 *B*). APC labeled with fluorescein in the active site (fl-APC) was incubated with EA.hy926 cells in the presence or absence of either plasma EPCR or rsEPCR. The EPCR concentration dependence for inhibition of APC binding to the cells was similar for both soluble forms of EPCR. This observation indicates that the affinity of plasma EPCR for binding APC is similar to that previously determined for the rsEPCR-APC binding interaction ($K_{d_{app}}$ ~ 30 nM) (11).

While rsEPCR has little effect on protein C activation in a soluble system (12), membrane-bound EPCR has a very potent ability to facilitate activation on cell surfaces (8). The current data demonstrating the existence of a circulating form of

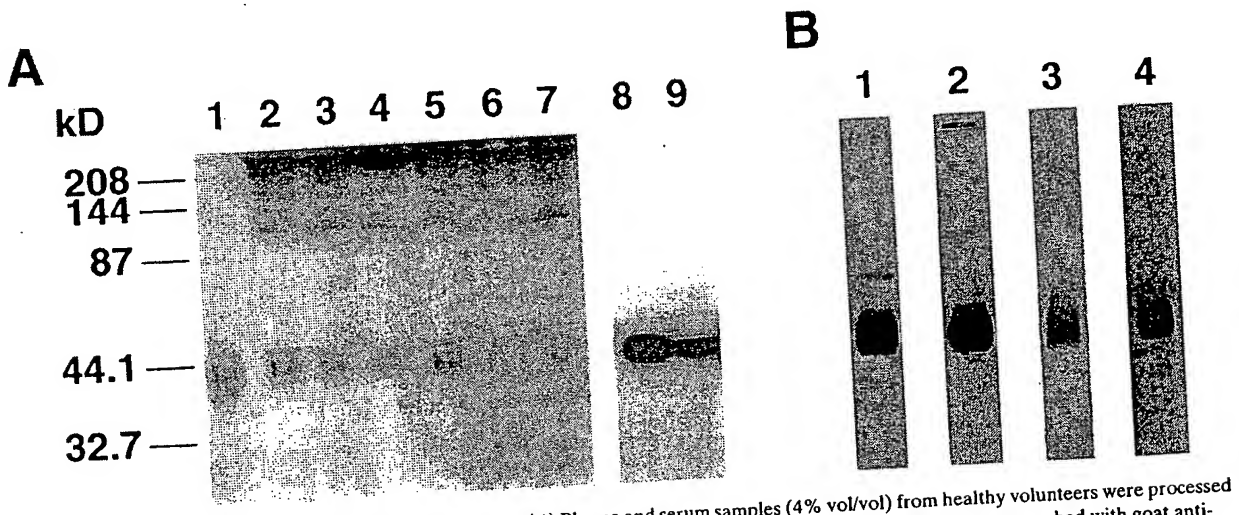


Figure 1. Detection of EPCR antigen in plasma and serum. (*A*) Plasma and serum samples (4% vol/vol) from healthy volunteers were processed for SDS-PAGE on 10% gels under nonreducing conditions, were transferred to membranes, and the membranes were probed with goat anti-rsEPCR polyclonal antibody (lanes *2–4*, serum; lanes *5–7*, plasma). Lane *1* contains rsEPCR (0.2 ng) Bound antibody was detected with mouse anti–goat IgG and an enhanced chemiluminescence detection system. Plasma samples from two healthy volunteers were immunoadsorbed with 1495 AffiGel-10 resin (lanes *8* and *9*). The washed resin was eluted and processed for SDS-PAGE under reducing conditions. Western blotting was done using biotin goat anti-rsEPCR as a probe. (*B*) Plasma EPCR purity was determined from silver-stained SDS-PAGE 10% gels (lanes *1*, reduced; 2, nonreduced) and Western blots of membranes probed with biotinylated goat anti-rsEPCR (lanes *3*, reduced; *4*, nonreduced). The molecular masses of the standards in kilodaltons are indicated on the left.

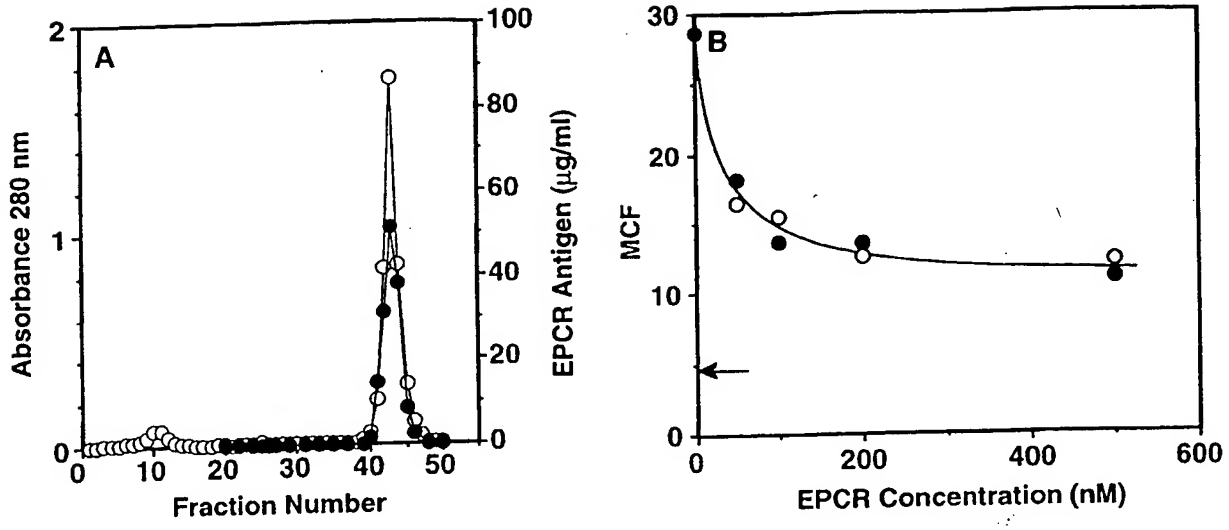


Figure 2. Soluble plasma EPCR binds to human protein C and APC. (*A*) A protein C affinity column was prepared by applying protein C (3 mg) to an HPC4 mAb AffiGel-10 immunoaffinity resin in buffer containing 3 mM $CaCl_2$, 0.6 mM $MgCl_2$. The column was washed, and plasma EPCR was applied at fraction 19. The column was washed and eluted with buffer containing 5 mM EDTA starting at fraction 35. Absorbance at 280 nm (○) and EPCR antigen (●) was determined for the indicated fractions. EPCR antigen was determined by ELISA as described. (*B*) EA.hy926 cells were incubated with 60 nM fl-APC in the presence of 0–500 nM rsEPCR (●) or plasma EPCR (○) for 30 min on ice. The cells were washed, and cell-bound fluorescence was determined by flow cytometry as described. The intrinsic cell fluorescence in the absence of added fl-APC is indicated by the arrow. The mean cell fluorescence (*MCF*) plotted represents the average of duplicate MCF determinations.

EPCR capable of binding protein C and APC suggested that plasma EPCR has the potential to alter cell-surface activation of protein C. The thrombin-dependent activation of an approximately physiological level of protein C (0.1 μM) on EA.hy926 cells was inhibited by excess rsEPCR almost to the level of that observed with the anti-rsEPCR 1494 mAb that blocks the EPCR-protein C binding interaction (Fig. 3 *A*). Previous studies have demonstrated that rsEPCR has no effect on APC amidolytic activity using small synthetic substrates (12). The plasma EPCR was slightly more effective in its ability to

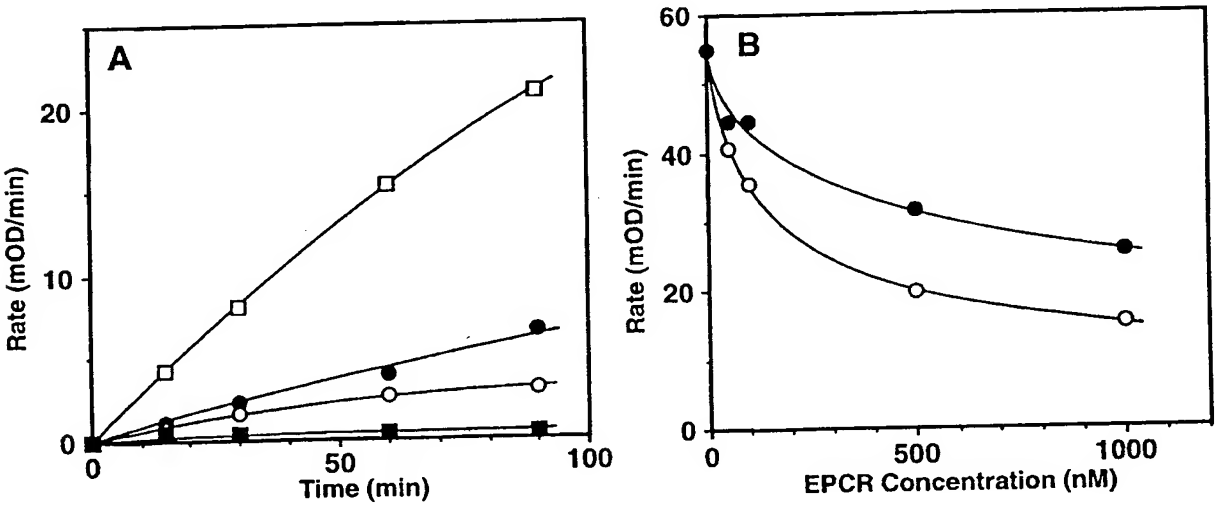


Figure 3. Soluble plasma EPCR and rsEPCR inhibit protein C activation on cell surfaces. (*A*) EA.hy926 cell monolayers were preincubated for 15 min at room temperature with 0.1 μM protein C alone (□), with 1 μM rsEPCR (●), or with 2 μg/ml 1494 mAb (○). Protein C activation was initiated by the addition of thrombin (2 nM final), and the reactions were stopped at the indicated times. APC was determined with an amidolytic assay as described, and the activity rates in mOD/min are plotted for each time point. Control wells without added thrombin were included (■). Each data point represents the average of triplicate well determinations. (*B*) EA.hy926 cell monolayers were preincubated for 15 min at room temperature with 0.1 μM protein C and the indicated concentrations of plasma EPCR (○) or rsEPCR (●). Thrombin (final 2 nM) was added, and the activation proceeded for 60 min at room temperature. The supernatants were added to a mixture of antithrombin and heparin, and APC activities (mOD/min) were determined with an amidolytic assay. Each data point represents the average of triplicate well determinations.

inhibit cell-surface protein C activation on the EA.hy926 cells relative to the rsEPCR (Fig. 3 *B*).

In a one-stage Factor Xa clotting assay, purified plasma and soluble recombinant EPCR inhibited the APC prolongation of clotting times similarly (Fig. 4). Inhibition of APC anticoagulant activity by rsEPCR had been observed previously (12). As expected, the 1496 mAb reversed this effect by blocking the APC plasma-EPCR binding interaction.

Discussion

The current studies demonstrate that a soluble form of EPCR circulates in plasma. In our healthy donor population, the plasma EPCR level was ~ 100 ng/ml, and it appeared to be a single antigen species of ~ 43,000 D. Subsequent purification of the soluble EPCR from plasma and functional studies determined that it was capable of binding both protein C and APC with an affinity similar to intact membrane-bound EPCR. The in vitro studies using an endothelial cell line demonstrated that plasma EPCR inhibited protein C activation at near physiological concentrations of protein C and thrombin. In addition, direct addition of purified plasma EPCR to plasma resulted in inhibition of APC anticoagulant activity that was reversed with monoclonal antibodies to rsEPCR.

The identification of the purified plasma protein as being EPCR was based on comparison with the properties of rsEPCR. These proteins both reacted with the same battery of monoclonal and polyclonal antibodies, had the same amino-terminal sequence, bound to immobilized protein C in a Ca^{2+}-dependent fashion, and blocked protein C activation and APC anticoagulant activity with similar dose–response curves. In addition, the affinities of both protein C and APC for rsEPCR and plasma EPCR are similar to the affinity of intact membrane-bound EPCR. These properties are, to our knowledge, unique to EPCR.

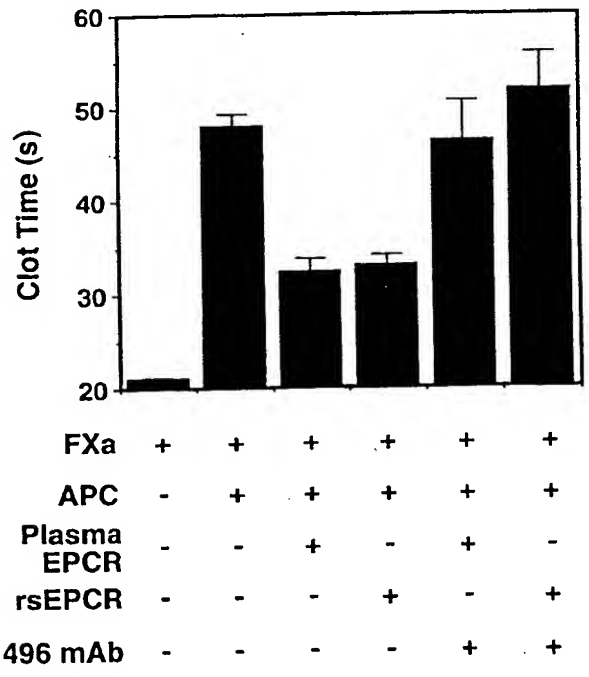


Figure 4. Soluble plasma EPCR inhibits APC anticoagulant activity. The anticoagulant activity of APC (25 nM) was determined with a one-stage Factor Xa clotting assay in the presence of 460 nM plasma EPCR or rsEPCR. The effect was reversed when either soluble EPCR was preincubated for 5 min with 42 μg/ml of 1496 mAb, which blocks binding of APC to EPCR. The data represent the average of four to six determinations±SD.

Previous studies from this laboratory demonstrated that membrane-bound EPCR expressed on endothelial cells augments protein C activation by a factor of three- to fivefold (8), whereas the current data demonstrate that the soluble form of EPCR purified from plasma inhibits protein C activation and APC anticoagulant activity. This contradiction predicts that EPCR could modulate the protein C pathway in several ways. First, in the larger vessels where thrombomodulin expression and density is reduced relative to the microcirculation, EPCR expression is correspondingly increased (43a) and may play a critical role in capturing the protein C substrate from the circulation and presenting it to the thrombin–thrombomodulin complex for activation. This hypothesis is supported by in vitro observations that both the EA.hy926 endothelial cell line and human umbilical vein endothelial cells have at least six times more surface-expressed EPCR antigen than thrombomodulin (Kurosawa, S., and D. Stearns-Kurosawa, unpublished observations). In the microcirculation where thrombomodulin density is high and EPCR is low, one would predict little influence on protein C activation. Finally, circulating soluble EPCR may reduce the generation of APC and the ability of APC to inactivate Factor Va. Previous studies with rsEPCR showed little effects of protein S on the APC-EPCR interaction with Factor Va (11, 12), but more complete studies are needed to determine the effect of the plasma EPCR-APC species on an assembled prothrombinase complex on cell surfaces.

In a healthy individual, the soluble EPCR levels are ~ 2.5 nM, a concentration well below both the $K_{d_{app}}$ (~ 30 nM) and the 80 nM protein C concentration in the circulation. Both of the effects of soluble plasma EPCR (inhibition of APC anticoagulant activity and protein C activation) required considerably higher concentrations than that present in normal plasma, leaving the question of the physiological role of the plasma EPCR uncertain. In preliminary studies we have found patients with soluble EPCR levels that exceed 40 nM (manuscript in preparation). Thus, if the local concentration near the endothelial cell surface exceeds the systemic concentration, the soluble EPCR concentration could reach levels that could attenuate both APC generation and activity, possibly contributing to a thrombotic risk.

A soluble form of a receptor can be produced by proteolytic cleavage of the membrane-bound receptor, or by alternative splicing mechanisms. Proteolysis at the membrane surface releases soluble thrombomodulin (18), and receptors for TNF, IL-1, IL-2, and PDGF (44). Soluble receptors have a multitude of potential functions, including acting as antagonists of the membrane receptor, stabilizing the ligand, initiating ligand-mediating signaling, downmodulation of the membrane form, and binding to receptor inhibitors to facilitate indirectly receptor–ligand activity. The latter mechanism is used by the IL-1 receptor system in which the soluble isoforms of both IL-1 receptors are generated by proteolytic cleavage, and tightly regulate the responsiveness to IL-1α and IL-1β (45). The bovine EPCR genomic structure contains an alterna-

tive splicing site that would code for a soluble protein truncated just before the transmembrane domain (46). Interestingly, soluble IL-6 receptors appear to be generated by both proteolytic and alternative splicing mechanisms (47–49).

Immunohistochemical studies have indicated that EPCR is located primarily on endothelium of large vessels, and is barely detectable in capillaries (43a). If the plasma EPCR is derived from membrane-bound EPCR, changes in plasma EPCR levels may serve as a marker of large vessel disease processes. In this sense, plasma EPCR may serve as an interesting comparison to plasma thrombomodulin levels, which have been shown to be modulated in a variety of disease states, but which would reflect both large and small vessel disease processes. The possibility of an alternatively spliced form of EPCR contributing to the plasma pool would have to be more rigorously tested before these studies would be interpretable.

The previous observation that the rsEPCR-APC interaction blocks APC anticoagulant activity without modulating reactivity with plasma proteinase inhibitors (12) is indicative of a change in macromolecular specificity rather than simple enzyme inhibition. This raises the possibility that the soluble plasma EPCR–APC complex has an alternative substrate, and the soluble form of EPCR may provide the function systemically. This is reminiscent of the specificity switch induced in thrombin by binding to thrombomodulin in which the coagulant activities are lost with a subsequent gain in protein C activation and anticoagulant activity (1, 10). While the exact role of plasma EPCR in vivo is unknown, it was demonstrated to be capable of modulating both the generation and the activity of APC in vitro. Further studies are needed to determine whether the soluble isoform of EPCR contributes to pathologic processes involving the protein C pathway.

Acknowledgments

The authors thank Steve Carpenter for tissue culture assistance; Tom Norton, Barbara Carpenter, and Teresa Burnett for technical assistance; Gary Ferrell for production of antibodies; Jeff Box for assistance with the figures; and Karen Deatherage for preparation of the final manuscript.

These studies were supported by grants awarded from the National Heart, Lung, and Blood Institute of the National Institutes of Health (grants P01 HL54804 and P50 HL54502). C.T. Esmon is an investigator of the Howard Hughes Medical Institute.

References

1. Esmon, C.T., and H.P. Schwarz. 1995. An update on clinical and basic aspects of the protein C anticoagulant pathway. *Trends Cardiovasc. Med.* 5:141–158.

2. Reitsma, P.H., F. Bernardi, R.G. Doig, S. Gandrille, J.S. Greengard, H. Ireland, M. Krawczak, B. Lind, G.L. Long, S.R. Poort, et al. 1995. Protein C deficiency: a database of mutations, 1995 update. *Thromb. Haemostasis.* 73:876–879.

3. Rivard, G.E., M. David, C. Farrell, and H.P. Schwarz. 1995. Treatment of purpura fulminans in meningococcemia with protein C concentrate. *J. Pediatr.* 126:646–652.

4. Gerson, W.T., J.D. Dickerman, E.G. Bovill, and E. Golden. 1993. Severe acquired protein C deficiency in purpura fulminans associated with disseminated intravascular coagulation: treatment with protein C concentrate. *Pediatrics.* 91:418–422.

5. Taylor, F.B., Jr., A. Chang, C.T. Esmon, A. D'Angelo, S. Vigano-D'Angelo, and K.E. Blick. 1987. Protein C prevents the coagulopathic and lethal effects of *E. coli* infusion in the baboon. *J. Clin. Invest.* 79:918–925.

6. Hancock, W.W., A. Tsuchida, H. Hau, N.M. Thomson, and H.H. Salem. 1992. The anticoagulants protein C and protein S display potent anti-inflammatory and immunosuppressive effects relevant to transplant biology and therapy. *Transplant. Proc.* 24:2302–2303.

7. Fukudome, K., and C.T. Esmon. 1994. Identification, cloning and regulation of a novel endothelial cell protein C/activated protein C receptor. *J. Biol. Chem.* 269:26486–26491.

8. Stearns-Kurosawa, D.J., S. Kurosawa, J.S. Mollica, G.L. Ferrell, and C.T. Esmon. 1996. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. *Proc. Natl. Acad. Sci. USA.* 93:10212–10216.

9. Esmon, C.T., and W.G. Owen. 1981. Identification of an endothelial cell cofactor for thrombin-catalyzed activation of protein C. *Proc. Natl. Acad. Sci. USA.* 78:2249–2252.

10. Dittman, W.A. 1991. Thrombomodulin: biology and potential cardiovascular applications. *Trends Cardiovasc. Med.* 1:331–336.

11. Fukudome, K., S. Kurosawa, D.J. Stearns-Kurosawa, X. He, A.R. Rezaie, and C.T. Esmon. 1996. The endothelial cell protein C receptor: cell surface expression and direct ligand binding by the soluble receptor. *J. Biol. Chem.* 271:17491–17498.

12. Regan, L.M., D.J. Stearns-Kurosawa, S. Kurosawa, J. Mollica, K. Fukudome, and C.T. Esmon. 1996. The endothelial cell protein C receptor: inhibition of activated protein C anticoagulant function without modulation of reaction with proteinase inhibitors. *J. Biol. Chem.* 271:17499–17503.

13. Jackman, R.W., D.L. Beeler, L. Fritze, G. Soff, and R.D. Rosenberg. 1987. Human thrombomodulin gene is intron depleted: nucleic acid sequences of the cDNA and gene predict protein structure and suggest sites of regulatory control. *Proc. Natl. Acad. Sci. USA.* 84:6425–6429.

14. Wen, D., W.A. Dittman, R.D. Ye, L.L. Deaven, P.W. Majerus, and J.E. Sadler. 1987. Human thrombomodulin: complete cDNA sequence and chromosome localization of the gene. *Biochemistry.* 26:4350–4357.

15. Suzuki, K., H. Kusumoto, Y. Deyashiki, J. Nishioka, I. Maruyama, M. Zushi, S. Kawahara, G. Honda, S. Yamamoto, and S. Horiguchi. 1987. Structure and expression of human thrombomodulin, a thrombin receptor on endothelium acting as a cofactor for protein C activation. *EMBO (Eur. Mol. Biol. Organ.) J.* 6:1891–1897.

16. Galvin, J.B., S. Kurosawa, K. Moore, C.T. Esmon, and N.L. Esmon. 1987. Reconstitution of rabbit thrombomodulin into phospholipid vesicles. *J. Biol. Chem.* 262:2199–2205.

17. Parkinson, J.F., B.W. Grinnell, R.E. Moore, J. Hoskins, C.J. Vlahos, and N.U. Bang. 1990. Stable expression of a secretable deletion mutant of recombinant human thrombomodulin in mammalian cells. *J. Biol. Chem.* 265:12602–12610.

18. Ye, J., N.L. Esmon, C.T. Esmon, and A.E. Johnson. 1991. The active site of thrombin is altered upon binding to thrombomodulin: two distinct structural changes are detected by fluorescence, but only one correlates with protein C activation. *J. Biol. Chem.* 266:23016–23021.

19. Lu, R., N.L. Esmon, C.T. Esmon, and A.E. Johnson. 1989. The active site of the thrombin-thrombomodulin complex: a fluorescence energy transfer measurement of its distance above the membrane surface. *J. Biol. Chem.* 264: 12956–12962.

20. Ye, J., L. Liu, C.T. Esmon, and A.E. Johnson. 1992. The fifth and sixth growth factor-like domains of thrombomodulin bind to the anion exosite of thrombin and alter its specificity. *J. Biol. Chem.* 267:11023–11028.

21. Hofsteenge, J., H. Taguchi, and S.R. Stone. 1986. Effect of thrombomodulin on the kinetics of the interaction of thrombin with substrates and inhibitors. *Biochem. J.* 237:243–251.

22. Mathews, I.I., K.P. Padmanabhan, and A. Tulinsky. 1994. Structure of a nonadecapeptide of the fifth EGF domain of thrombomodulin complexed with thrombin. *Biochemistry.* 33:13547–13552.

23. Esmon, C.T., N.L. Esmon, and K.W. Harris. 1982. Complex formation between thrombin and thrombomodulin inhibits both thrombin-catalyzed fibrin formation and factor V activation. *J. Biol. Chem.* 257:7944–7947.

24. Sadler, J.E., S.R. Lentz, J.P. Sheehan, M. Tsiang, and Q. Wu. 1993. Structure-function relationships of the thrombin-thrombomodulin interaction. *Haemostasis.* 23:183–193.

25. Bourin, M.C., and U. Lindahl. 1993. Glycosaminoglycans and the regulation of blood coagulation. *Biochem. J.* 289:313–330.

26. Rezaie, A.R., S.T. Cooper, F.C. Church, and C.T. Esmon. 1995. Protein C inhibitor is a potent inhibitor of the thrombin-thrombomodulin complex. *J. Biol. Chem.* 270:25336–25339.

27. Takano, S., S. Kimura, S. Ohdama, and N. Aoki. 1990. Plasma thrombomodulin in health and diseases. *Blood.* 76:2024–2029.

28. Ishii, H., and P.W. Majerus. 1985. Thrombomodulin is present in human plasma and urine. *J. Clin. Invest.* 76:2178–2181.

29. Tanaka, A., H. Ishii, S. Hiraishi, M. Kazama, and H. Maezawa. 1991. Increased thrombomodulin values in plasma of diabetic men with microangiopathy. *Clin. Chem.* 27:269–272.

30. Takahashi, H., M. Hanano, K. Wada, W. Tatewaki, H. Niwano, J. Tsubouchi, M. Nakano, T. Nakamura, and A. Shibata. 1991. Circulating thrombomodulin in thrombotic thrombocytopenic purpura. *Am. J. Hematol.* 38:174–177.

31. Asakura, H., H. Jokaji, M. Saito, C. Uotani, I. Kumabashiri, E. Morishita, M. Yamazaki, and T. Matsuda. 1991. Plasma levels of soluble thrombomodulin increase in cases of disseminated intravascular coagulation with organ

failure. *Am. J. Hematol.* 38:281–287.

32. Wada, H., M. Ohiwa, T. Kaneko, S. Tamaki, M. Tanigawa, S. Shirakawa, M. Koyama, T. Hayashi, and K. Suzuki. 1992. Plasma thrombomodulin as a marker of vascular disorders in thrombotic thrombocytopenic purpura and disseminated intravascular coagulation. *Am. J. Hematol.* 39:20–24.

33. Takahashi, H., S. Ito, M. Hanano, K. Wada, H. Niwano, Y. Seki, and A. Shibata. 1992. Circulating thrombomodulin as a novel endothelial cell marker: comparison of its behavior with von Willebrand factor and tissue-type plasminogen activator. *Am. J. Hematol.* 41:32–39.

34. Ohdama, S., O. Matsubara, and N. Aoki. 1994. Plasma thrombomodulin in Wegener's granulomatosis as an indicator of vascular injuries. *Chest.* 106: 666–671.

35. Mather, T., V. Oganessyan, P. Hof, R. Huber, S. Foundling, C. Esmon, and W. Bode. 1996. The 2.8 Å crystal structure of Gla-domainless activated protein C. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:6822–6831.

36. Esmon, C.T., N.L. Esmon, B.F. Le Bonniec, and A.E. Johnson. 1993. Protein C activation. *Methods Enzymol.* 222:359–385.

37. Owen, W.G., C.T. Esmon, and C.M. Jackson. 1974. The conversion of prothrombin to thrombin. I. Characterization of the reaction products formed during the activation of bovine prothrombin. *J. Biol. Chem.* 249:594–605.

38. Esmon, C.T. 1977. Factors regulating the inhibition of thrombin by antithrombin III. *In* Chemistry and Biology of Thrombin. R.L. Lundblad, J.W. Fenton II, and K.G. Mann, editors. Ann Arbor Science Publishers, Inc., Ann Arbor, MI. 403–411.

39. Stearns, D.J., S. Kurosawa, P.J. Sims, N.L. Esmon, and C.T. Esmon. 1988. The interaction of a Ca^{2+} dependent monoclonal antibody with the protein C activation peptide region: evidence for obligatory Ca^{2+} binding to both antigen and antibody. *J. Biol. Chem.* 263:826–832.

40. Edgell, C.S., C.C. McDonald, and J.B. Graham. 1983. Permanent cell line expressing human factor VIII-related antigen established by hybridization. *Proc. Natl. Acad. Sci. USA.* 80:3734–3737.

41. Bock, P.E. 1988. Active site selective labeling of serine proteases with spectroscopic probes using thioester peptide chloromethyl ketones: demonstration of thrombin labeling using N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl. *Biochemistry.* 27:6633–6639.

42. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680–685.

43. Quehenberger, P., U. Loner, S. Kapiotis, S. Handler, B. Schneider, J. Huber, and W. Speiser. 1996. Increased levels of activated factor VII and decreased plasma protein S activity and circulating thrombomodulin during use of oral contraceptives. *Thromb. Haemostasis.* 76:729–734.

43a. Laszik, Z., A. Mitro, F.B. Taylor, Jr., G. Ferrell, and C.T. Esmon. 1997. The human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. *Circulation.* In press.

44. Heaney, M.L., and D.W. Golde. 1996. Soluble cytokine receptors. *Blood.* 87:847–857.

45. Arend, W.P., M. Malyak, M. Smith, Jr., T.D. Whisenand, J.L. Slack, J.E. Sims, J.G. Giri, and S.K. Dower. 1994. Binding of IL-1α, IL-1β, and IL-1 receptor antagonist by soluble IL-1 receptors and levels of soluble IL-1 receptors in synovial fluids. *J. Immunol.* 153:4766–4774.

46. Fukudome, K., and C.T. Esmon. 1995. Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR). The structural and functional conservation in human, bovine, and murine EPCR. *J. Biol. Chem.* 270:5571–5577.

47. Mullberg, J., W. Oberthur, F. Lottspeich, E. Mehl, E. Dittrich, L. Graeve, P.C. Heinrich, and S. Rose-John. 1994. The soluble human IL-6 receptor. Mutational characterization of the proteolytic cleavage site. *J. Immunol.* 152:4958–4968.

48. Lust, J.A., K.A. Donovan, M.P. Kline, P.R. Greipp, R.A. Kyle, and N.J. Maihle. 1992. Isolation of an mRNA encoding a soluble form of the human interleukin-6 receptor. *Cytokine.* 4:96–100.

49. Horiuchi, S., Y. Koyanagi, Y. Zhou, H. Miyamoto, Y. Tanaka, M. Waki, A. Matsumoto, M. Yamamoto, and N. Yamamoto. 1994. Soluble interleukin-6 receptors released from T cell or granulocyte/macrophage cell lines and human peripheral blood mononuclear cells are generated through an alternative splicing mechanism. *Eur. J. Immunol.* 24:1945–1948.

Oral Anticoagulants

Mechanism of Action, Clinical Effectiveness, and Optimal Therapeutic Range

Jack Hirsh, MD, FCCP, Chair; James E. Dalen, MD, FCCP; David R. Anderson, MD; Leon Poller, MD; Henry Bussey, PharmD; Jack Ansell, MD; Daniel Deykin, MD; and John T. Brandt, MD

(CHEST 1998; 114:445S–469S)

The optimal therapeutic range for oral anticoagulant therapy was reviewed by the Committee on Antithrombotic Therapy of the American College of Chest Physicians (ACCP) and the National Heart, Lung, and Blood Institute in 1986, in 1989, in 1992, and again in 1995.[1] A recommendation was made that the intensity of warfarin (Coumadin) treatment should be reduced for many indications.[1] Since then, new clinical trials have been published that support these recommendations and the optimal therapeutic range for many indications has been clarified (Table 1).

Progress has been made in the control of oral anticoagulant therapy because the importance of reporting the prothrombin time (PT) results as an international normalized ratio (INR) is now recognized. A recommendation of an INR of 2.0 to 3.0 is made for most indications. The exceptions are some types of mechanical prosthetic heart valves (see chapter on "Antithrombotic Therapy in Patients With Mechanical and Biological Prosthetic Heart Valves", [page 602S]), postmyocardial infarction, and certain patients with thrombosis and the antiphospholipid syndrome, for which an INR of 2.5 to 3.5 is recommended. Results of a number of recent studies—two randomized studies, one subgroup analysis of a prospective cohort study, and a case-control study—indicate that the effectiveness of warfarin in atrial fibrillation is reduced when the INR falls below 2.0 and is essentially lost when the INR falls below 1.5. In contrast, the Thrombosis Prevention Trial,[2] a primary prevention study in men free of ischemic heart disease at entry, reported that warfarin is effective in reducing myocardial ischemic events (including fatal events) when used at a targeted INR of 1.3 to 1.8 (mean warfarin dose of 4.1 mg). The addition of low-dose aspirin to warfarin resulted in a further small benefit but at a risk of increased bleeding.

In summary, the results of recent studies (1) do not support the use of fixed low-dose warfarin therapy for the treatment of patients with acute myocardial infarction or atrial fibrillation, (2) indicate that the effectiveness of warfarin is reduced when the INR is < 2.0, and (3) indicate that adjusted-dose warfarin therapy produces some benefit at an INR of 1.3 to 2.0 when used for primary prevention, and that an INR of > 1.5 confers some benefit in patients with atrial fibrillation, although the benefit is clearly less than that which occurs with an INR of > 2.0.

Correspondence to: Jack Hirsh, MD, FCCP, Research Centre, Hamilton Civic Hospitals, 711 Concession, Hamilton, ON, Canada L8V 1C3

Mechanism of Action, Pharmacokinetics, and Pharmacodynamics of Warfarin

Oral anticoagulants produce their anticoagulant effect by interfering with the cyclic interconversion of vitamin K and its 2,3 epoxide (vitamin K epoxide). Vitamin K is a cofactor for the post-translational carboxylation of glutamate residues to γ-carboxyglutamates (Gla) on the N-terminal regions of vitamin K-dependent proteins.[3–8] The process of γ-carboxylation permits the coagulation proteins to undergo a conformational change[9–11] in the presence of calcium ions, a necessary requirement for calcium-dependent complexing of vitamin K-dependent proteins to their cofactors on phospholipid surfaces and for their biological activity. Carboxylation of vitamin K-dependent coagulation factors is catalyzed by a carboxylase that requires the reduced form of vitamin K (vitamin KH_2), molecular oxygen, and carbon dioxide. During this reaction, the vitamin KH_2 is oxidized to vitamin K epoxide, which is recycled to vitamin K by vitamin K epoxide reductase, which in turn is reduced to vitamin KH_2 by vitamin K reductase. The vitamin K antagonists exert their anticoagulant effect by inhibiting vitamin K epoxide reductase[3–5] and possibly vitamin K reductase.[4] This process leads to the depletion of vitamin KH_2 and limits the γ-carboxylation of the vitamin K-dependent coagulant proteins (prothrombin, factor VII, factor IX, and factor X). In addition, the vitamin K antagonists limit the carboxylation of the regulatory anticoagulant proteins (protein C and protein S), and as a result impair their function. By inhibiting the cyclic conversion of vitamin K, oral anticoagulants result in the hepatic production and secretion of partially carboxylated and descarboxylated proteins.[12,13] Reduction of the number of Gla residues on the prothrombin molecule from the normal complement of 10–13 to 9 Gla residues results in a 30% reduction in coagulant activity, while reduction to < 6 residues results in a loss of $> 95\%$ of coagulant activity.[14,15] A possible association with osteoporosis between warfarin use and osteoporosis in postmenopausal women has been suggested, but definitive evidence for such an association is lacking. The anticoagulant effect of warfarin can be overcome by low doses of vitamin K_1, because the oxidized form of the vitamin can be reduced through a different warfarin-resistant vitamin K reductase enzyme system that is operative at high tissue concentrations of vitamin K_1 (Fig 1). It is for this reason that patients become warfarin resistant for up to a week or more if given large doses of vitamin K_1.

Effects of Warfarin on Bone Metabolism

Gla proteins synthesized in bone include osteocalcin, protein S, and matrix Gla protein.[16–18] Warfarin interferes with the carboxylation of these proteins and inhibits the effect of vitamin K in osteoblasts.[19] These effects of warfarin could be responsible for bone abnormalities that can occur in neonates from women treated with warfarin

Table 1—*Recommended Therapeutic Range for Oral Anticoagulant Therapy*

Indication	INR
Prophylaxis of venous thrombosis (high-risk surgery)	
Treatment of venous thrombosis	
Treatment of pulmonary embolism	
Prevention of systemic embolism	
Tissue heart valves	2.0–3.0
AMI (to prevent systemic embolism)*	
Valvular heart disease	
Atrial fibrillation	
Mechanical prosthetic valves (high risk)	2.5–3.5
Bileaflet mechanical valve in aortic position	2.0–3.0

*If oral anticoagulant therapy is elected to prevent recurrent myocardial infarction, an INR of 2.5 to 3.5 is recommended, consistent with Food and Drug Administration recommendations.

during pregnancy.[20,21] However, there is no evidence that warfarin has adverse effects on bone metabolism when administered to children or adults.

Pharmacokinetics and Pharmacodynamics of Warfarin

Warfarin (a 4-hydroxy compound) is the most widely used oral anticoagulant in North America. It has a predictable onset and duration of action and excellent bioavailability.[22,23] Warfarin is almost always administered by the oral route, although an injectable preparation is available in the United States. Warfarin is a racemic mixture of roughly equal amounts of two optically active isomers, the R and S forms. Warfarin is rapidly absorbed from the GI tract and reaches maximal blood concentrations in healthy volunteers in 90 min.[22,24] Racemic warfarin has a half-life of 36 to 42 h;[25] it circulates bound to plasma proteins (mainly albumen) and rapidly accumulates in the liver where the two isomers are metabolically transformed by different pathways.[25] The dose response relationship of warfarin differs between healthy subjects[26] and can vary to a much greater extent among sick patients. Because of the variations in dose response in individual patients during the course of anticoagulant therapy, their anticoagulant dosage must be monitored closely to prevent overdosing or underdosing.

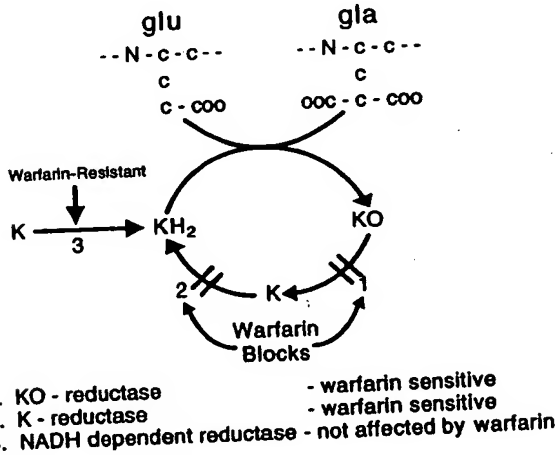


FIGURE 1. Vitamin K_1 is reduced to vitamin KH_2 by two warfarin-sensitive enzymes (KO-reductase to K-reductase) and the NADH dependent reductase system that is insensitive to warfarin.

The dose response to warfarin is influenced by both pharmacokinetic factors (due to differences in absorption or metabolic clearance of warfarin) and pharmacodynamic factors (due to differences in the hemostatic response to given concentrations of warfarin). Technical factors also contribute to the apparent variability in response, including inaccuracies in laboratory testing and reporting, poor patient compliance, and poor communication between patient and physician. Occasionally, the cause of the variable response within individual patients remains unexplained.

Drugs can influence the pharmacokinetics of warfarin by reducing its absorption from the intestine or by altering its metabolic clearance. The anticoagulant effect of warfarin is reduced by cholestyramine, which impairs its absorption. It can be potentiated by drugs that inhibit the metabolic clearance of warfarin either through stereoselective or nonselective pathways.[26–28] Stereoselective interactions can affect the oxidative metabolism of the S-isomer or R-isomer of warfarin.[27,28] Inhibition of metabolism of S-warfarin is more important clinically because this isomer is five times more potent as a vitamin K antagonist than the R-form.[27,28] Therefore, drugs that inhibit clearance of the S-isomer prolong the PT to a much greater degree than drugs that inhibit the metabolic clearance of the R-isomer. The clearance of S-warfarin is inhibited by phenylbutazone,[29,30] sulfinpyrazone,[31] metronidazole,[32] and trimethoprim-sulfamethoxazole,[33] all of which have been documented to potentiate the effect of warfarin on the PT. In contrast, drugs such as cimetidine and omeprazole, which only inhibit the metabolic clearance of the R-isomer, have only moderate potentiating effects on the PT in patients treated with oral anticoagulants.[28,29,34] Amiodarone inhibits the metabolic clearance of both the S- and R-isomers and has an important potentiating effect on the anticoagulant effect of warfarin.[35] The anticoagulant effect of warfarin is inhibited by drugs such as barbiturates,[33] rifampicin,[35] and carbamazepine[33] that increase its metabolic clearance by inducing activity of hepatic mixed oxidases. Long-term alcohol use has the potential to increase the clearance of warfarin by hepatic enzyme induction, although studies have shown that even relatively high wine consumption does not influence the PT in subjects treated with warfarin.[30] For a more thorough discussion of the effect of enzyme induction on warfarin therapy, the reader is referred to a recent critical review of the literature[36] (Table 2).

The pharmacodynamics of warfarin are affected by many factors that can influence its anticoagulant effect.

Hereditary resistance to warfarin has been described in rats[37] and humans.[38,39] The affected humans require warfarin in doses that are 5- to 20-fold higher than average to achieve an anticoagulant effect. This disorder is thought to

Table 2—*Enzyme-Inducing Drug Interactions With Warfarin

Inducing Agent	Isoenzyme Induced**	Expected Onset, d	Anticipated Dosage Adjustments, %	Expected Offset, d	Predictive Confidence
Carbamazepine	CYP3A4	10–35	↑ 100	42	+++
Barbiturate†	CYP3A	7–30	↑ 12.5–25	> 42	++++
Phenytoin	Nonspecific	NA	↑ ↓	NA	+
Rifampin	CYP3A4	< 7	↑ 100–200	21	++++
Griseofulvin‡	?	60	↑ 40	NA	+++
Nafcillin	NA	< 7	↑ 100–400	7–28	+++
Dicloxacillin	NA	< 7	↑ 2–30	NA	+++
Aminoglutethimide§	CYP2B1	14	↑ 50–75	14	+++
Smoking	CYP1A1, 1A2	NA	↑‖	NA	+++
Alcohol	CYP2E1				
41–54 g¶			↔		++++
250 g#		NA	↑‖	NA	+++

*Upward arrow = an increase in warfarin dosage is anticipated with initiation of the inducing agent; downward arrow = a decrease in warfarin dosage is anticipated with initiation of inducing agent; side-to-side arrow = no change in warfarin dosage appears necessary based on available data; NA = not available.

**Information regarding induction of cytochrome-450 isoenzymes is limited; current literature supports specific isoenzyme induction by the listed agent.

†Class effect, although time course and extent may vary with the individual barbiturate.

‡Interaction is more likely with the ultramicrocrystalline formulation of griseofulvin.

§Dose-response relationship, so that 250 mg 4 times a day showed greater induction than 125 mg 4 times a day.

‖Warfarin clearance increased, but a corresponding change in PT was not reported. See text for further details.

¶Represents ingestion of 41 to 54 g of ethanol consumed either as a single dose or daily for 21 days.

#Represents ingestion of large amounts of ethanol (250 g) consumed daily for > 3 months.

be caused by an altered affinity of the receptor for warfarin since the plasma warfarin levels required to achieve an anticoagulant effect are much higher than average. There are also rare anecdotal reports of acquired resistance to warfarin that cannot be explained by lack of compliance, by excessive vitamin K ingestion, or by the use of interacting drugs.

Subjects receiving long-term warfarin therapy are sensitive to fluctuating levels of dietary vitamin K[40,41] that is obtained predominantly from phylloquinone in plant material.[41] Phylloquinone acts through the warfarin-insensitive pathway.[42] The phylloquinone content of a wide range of foodstuffs has been listed by Sadowski and associates.[43] Additionally, two lists of the vitamin K content of various foods are available on the Internet at <www.nal.usda.gov/fnic/foodcomp/Data/Other/vitk2.dat> and <www.wisconsin.4biznet/health/factor-v/vkchart1.htm>. It is possible that the seasonal effect of oral anticoagulant regimens reported by Manotti and associates[44] was the result of seasonal variation of intake of fresh vegetables.

Important fluctuations in vitamin K intake occur in both apparently healthy and sick subjects.[45] Increased intake of dietary vitamin K sufficient to reduce the anticoagulant response to warfarin[40] occurs in patients on weight reduction diets (rich in green vegetables or vitamin K-containing dieting supplements) and those treated with IV nutritional fluid supplements rich in vitamin K. The effects of warfarin can be potentiated in sick patients with poor vitamin K_1 intake (particularly if they are treated with antibiotics and IV fluids without vitamin K supplementation) and in states of fat malabsorption. Hepatic dysfunction also potentiates the response to warfarin through impaired synthesis of coagulation factors. Hypermetabolic states produced by fever or hyperthyroidism increase responsiveness to warfarin probably by increasing the catabolism of vitamin K-dependent coagulation factors.[46,47] Drugs can influence the pharmacodynamics of warfarin by inhibiting the synthesis of vitamin K-dependent coagulation factors, by increasing the metabolic clearance of vitamin K-dependent coagulation factors, and by interfering with other pathways of hemostasis (Table 3). The anticoagulant effect of warfarin is augmented by the second- and third-generation cephalosporins because these antibiotics inhibit the cyclic interconversion of vitamin K,[48,49] by thyroxine because this hormone increases the rate of metabolism of coagulation factors,[47] and by clofibrate through an unknown mechanism.[50] High doses of salicylates > 1.5 g and acetaminophen[51,52] have also been reported to augment the anticoagulant effect of warfarin,[53] possibly by warfarin-like action. Although heparin increases the anticoagulant effect of warfarin, it causes only a slight prolongation of the PT in therapeutic doses.

Drugs such as aspirin,[54] other nonsteroidal anti-inflammatory drugs,[55] high doses of penicillins,[56,57] and moxalactam[49] can increase the risk of warfarin-associated bleeding by inhibiting platelet function. Aspirin is the most important because of its widespread use and prolonged effect on hemostasis.[58] Aspirin and nonsteroidal anti-inflammatory drugs can also produce gastric erosions that increase the risk of serious upper GI bleeding.[57] The risk of clinically important bleeding is increased when high doses of aspirin are used in combination with high-intensity warfarin therapy (INR, 3.0 to 4.5).[54,59] A study in patients with prosthetic heart valves has also reported that low doses of

Table 3—*Drug and Food Interactions With Warfarin by Level of Supporting Evidence and Direction of Interaction

Level of Evidence	Potentiation	Inhibition	No Effect
I	Alcohol (if concomitant liver disease) amiodarone (anabolic steroids, cimetidine,† clofibrate, cotrimoxazole erythromycin, fluconazole, isoniazid [600 mg daily] metronidazole), miconazole, omeprazole, **phenylbutazone,** piroxicam, propafenone, propranolol,† **sulfinpyrazone (biphasic with later inhibition)**	Barbiturates, carbamazepine, chlordiazepoxide, cholestyramine, **griseofulvin,** nafcillin, rifampin, sucralfate, high vitamin K content foods/enteral feeds, large amounts of avocado	Alcohol, antacids, atenolol, bumetadine, enoxacin, famotidine, fluoxetine, ketorolac, metoprolol, naproxen, nizatidine, psyllium, ranitidine‡
II	Acetaminophen, chloral hydrate, ciprofloxacin, dextropropoxyphene, disulfiram, itraconazole, quinidine, phenytoin (biphasic with later inhibition), tamoxifen, tetracycline, flu vaccine	Dicloxacillin	Ibuprofen, ketoconazole
III	Acetylsalicylic acid, disopyramide, fluorouracil, ifosfhamide, ketoprofen, lovastatin, metozalone, moricizine, nalidixic acid, norfloxacin, ofloxacin, propoxyphene, sulindac, tolmetin, topical salicylates	Azathioprine, cyclosporine, etretinate, trazodone	
IV	Cefamandole, cefazolin, gemfibrozil, heparin, indomethacin, sulfisoxazole		Diltiazem, tobacco, vancomycin

*Drugs bold type are those that have supporting level I evidence from both patients and volunteers.
†In a small number of volunteer subjects, an inhibitory drug interaction occurred.
‡Level II evidence of potentiation in patients.

aspirin (100 mg/d)[60] increase the risk of minor bleeding and produced a nonsignificant trend to increase the risk of major bleeding.

Other drugs, including erythromycin[61] and some anabolic steroids,[62] potentiate the anticoagulant effect of warfarin through unknown mechanisms. Sulfonamides and many broad-spectrum antibiotics have the potential to augment the anticoagulant effect of warfarin by eliminating bacterial flora and, thereby, producing vitamin K deficiency, but these agents only potentiate the anticoagulant effect of warfarin in patients receiving a vitamin K-deficient diet.[63]

Wells and associates[64] performed a critical analysis of articles reporting a possible interaction between drugs or foods and warfarin. The entire Medline and Toxline databases were searched and each report was rated independently and received a score representing the level of assurance that a clinically important interaction had or had not occurred. Studies were assigned to a level 1 category if an interaction was considered to be highly probable, to a level 2 category if an interaction was probable, level 3 if it was possible, and level 4 if it was doubtful. Of the 751 citations retrieved, 172 were original reports and these are summarized in Table 3. Thirty-nine of the 81 different drugs and foods appraised were judged to have strong evidence: 17 potentiating warfarin effect, 10 inhibiting, and 12 producing no effect. Many other drugs either interact with oral anticoagulants or have been reported to alter the PT response to warfarin,[65,66] but in most of these reports, convincing evidence of a causal association is lacking. Although acetaminophen has never been considered to have a major impact on warfarin therapy, except perhaps at very high doses, a recent descriptive study noted that even low to moderate doses of acetaminophen (at least nine tablets per week) was associated with excessively elevated INR values. Whether this is a true association is uncertain, but it would be prudent to monitor the INR more frequently whenever a change is made in concomitant therapy. Similarly, it would be prudent to take special care when treatment with any new drug is necessary in patients who are being treated with oral anticoagulants and to monitor the PT more frequently during the initial stages of combined drug therapy with dose adjustments made, when appropriate.

Monitoring Oral Anticoagulant Therapy

The PT test is the most common method used for monitoring oral anticoagulant therapy.[67] The PT is responsive to depressions of three of the four vitamin K-dependent procoagulant clotting factors (factors II, VII, and X). These are reduced by warfarin at a rate proportionate to their respective half-lives. The PT is performed by adding calcium and thromboplastin to citrated plasma. The term "thromboplastin" traditionally refers to a phospholipid-protein extract of tissue, usually lung, brain, or placenta, that contains both the tissue factor and the phospholipid necessary to promote the activation of factor

X by factor VII. Recently developed thromboplastins utilize recombinant human tissue factor and defined phospholipid preparations. During the first few days of warfarin therapy, the PT reflects primarily the depression of factor VII, which has a half-life of only approximately 6 h. Subsequently, the test is prolonged also by depression of factors X and II. The "responsiveness" of a given thromboplastin to warfarin-induced changes in clotting factors mirrors the strength of the activation of factor X by factor VII as the levels of both of the clotting factors decrease. An "unresponsive" thromboplastin results in a lesser prolongation of the PT for a given reduction in vitamin K-dependent clotting factors than a more responsive thromboplastin.

Thromboplastins vary in their responsiveness to the anticoagulant effects of warfarin depending on their tissue of origin, phospholipid content, and method of preparation.[68-71] In the past, when the PT was expressed in seconds or as a simple ratio of the patient to normal PT, this led to confusion regarding the appropriate therapeutic range and dose of warfarin. For example, it takes a lower dose of warfarin to prolong the PT ratio to 2.0 if a responsive reagent (low international sensitivity index [ISI]) is used to measure the PT than it does if a less responsive (high ISI) reagent is used. During the 1980s, most laboratories in the United States used reagents with an ISI between 1.8 and 2.8, while many laboratories in Europe used more responsive reagents with ISIs of 1.0 to 1.4. The differences in the responsiveness of thromboplastins were mainly responsible for the clinically important differences in the dosage of oral anticoagulants used in different countries, as shown by Poller and Taberner.[71] Recognition of the clinical importance of these differences in reagent responsiveness led to the development and widespread adoption of the INR as a method to standardize monitoring of oral anticoagulant therapy.

In the study by Bussey and associates,[72] published in 1992, the ISI of the thromboplastins used in the United States varied between 1.4 and 2.8. Since that time, there has been an increase in the use of thromboplastins with lower ISI values (*ie*, more responsive thromboplastins) in the United States and Canada. For example, the recombinant human preparations, consisting of relipidated synthetic tissue factor, have ISI values of between 0.9 and 1.0.[73]

Since the efficacy of warfarin is reduced when the INR falls below 2.0, care should be taken to ensure that the INR is always maintained at 2.0 or higher when targeting an INR of 2.0 to 3.0; this can be ensured by aiming for an INR of about 2.5 when the targeted range is 2.0 to 3.0. Similarly, when the targeted INR is 2.5 to 3.5, it would be prudent to aim for an INR of about 3.0.

The Antithrombotic Effect of Warfarin

The conventional view is that the antithrombotic effect of warfarin reflects its anticoagulant effects and is mediated through its ability to inhibit thrombin generation by reducing the levels of the four vitamin K-dependent coagulation factors. There is evidence, however, that the reduction of prothrombin, and possibly factor X, is more important than reduction of factors VII and IX for the antithrombotic effect of warfarin. The evidence supporting this hypothesis is not definitive and comes from the following observations. First, the experiments of Wessler and Gitel[74] performed > 40 years ago using a stasis model of thrombosis in rabbits showed that the antithrombotic effects of warfarin require 6 days of treatment, whereas an anticoagulant effect is seen within 2 days. These data are consistent with an explanation that the antithrombotic effect of warfarin requires a reduction in the level of prothrombin, since this zymogen has a half-life of about 72 h, whereas the other K-dependent factors have half-lives of 6 to 24 h that contribute to and are responsible for warfarin's earlier anticoagulant effect. Second, in more recent experiments, in a rabbit model of tissue factor-induced intravascular coagulation, Zivelin et al[75] demonstrated that the protective effect of warfarin primarily reflected its ability to lower prothrombin levels. Thus, selective infusion of prothrombin, and to a lesser extent factor X, abolished the protective effects of warfarin in this model. In contrast, infusion of factor VII or factor IX had no effect. Third, using fibrinopeptide A (FPA) generation as an index of clot-associated thrombin activity, Patel and associates[76] demonstrated that clots formed from cord plasma (which contains about 50% of the prothrombin concentration of control adult plasma) generated significantly less FPA than clots formed from adult plasma. That the reduced activity of the clot from cord blood is due to the reduced levels of prothrombin in cord plasma was confirmed by the demonstration that (1) FPA generation by cord plasma clots became identical to that of adult plasma clots when the cord plasma was supplemented with adult levels of prothrombin, even though all of the vitamin K-dependent coagulation factors were reduced in cord plasma, and (2) that clot-induced FPA generation in prothrombin-depleted plasma is dependent on the level of prothrombin.

The biological rationale that warfarin exerts its antithrombotic effect through its ability to reduce prothrombin levels comes from the recent observations that clot-bound thrombin is an important mediator of clot growth.[77] Reduction in prothrombin levels decreases the amount of thrombin that can be generated and bound to fibrin and so reduces the thrombogenocity of the clot.[76] Thrombin bound to fibrin is enzymatically active and protected from inactivation by fluid-phase inhibitors. As a result, fibrin-bound thrombin acts as a reservoir that can locally amplify its own generation by activating platelets and factors V and VIII. There are two reasons why the concept that the antithrombotic effect of warfarin reflects its ability to lower prothrombin levels is of potential clinical importance. First, it provides a rationale for overlapping heparin with warfarin in the treatment of patients with thrombotic disease until the prothrombin level is lowered into the therapeutic range. Given that prothrombin has a half-life of about 60 h, an overlap of at least 4 days is necessary to achieve this. Second, it supports the contention of Furie and colleagues[78] that the levels of native prothrombin antigen are better than PTs for monitoring warfarin therapy because they more closely

reflect the antithrombotic activity of warfarin. Third, it supports the use of a maintenance dose of warfarin (approximately 5 mg) to initiate warfarin therapy, since based on a recent study,[79] the rate of lowering of prothrombin levels was similar when warfarin treatment was initiated with either a 5-mg or a 10-mg dose. However, the level of the anticoagulant protein, protein C, was reduced more rapidly and more patients were overanticoagulated (INR > 3.0) with the 10-mg loading dose.

At present, the clinical importance of these theoretical advantages of starting warfarin therapy with a 5-mg dose is uncertain and requires further study.

Standardization of the Prothrombin Time

The history of standardization of the PT has been reviewed by Poller[69] and by Kirkwood.[80] A standardized human brain thromboplastin reagent, the Manchester Comparative Reagent, was introduced in 1962 and used by nearly all hospitals in the United Kingdom for > 20 years until 1985. In 1977, the World Health Organization (WHO) designated a batch of human brain thromboplastin as the first international reference preparation (IRP) for thromboplastin.[69,80] The first IRP was later replaced by a new primary and three secondary reference thromboplastins. A calibration system was developed, based on the assumption that a linear relationship would exist between the logarithm of the PT obtained with the reference and test thromboplastins.[69,80,81] This calibration model adopted in 1982 is used to standardize the reporting of the PT by converting the PT ratio observed with the local thromboplastin into an INR, calculated as follows:

$$\text{INR} = \left(\frac{\text{Patient PT}}{\text{Mean Normal PT}}\right)^{\text{ISI}}$$

ie, $\log \text{INR} = \text{ISI} \times \log \text{observed PT ratio}$

where ISI is the power value that represents the International Sensitivity Index (ISI). A specially designed nomogram provides INR values from the prothrombin ratio value obtained with a thromboplastin reagent and its ISI without the need for calculations (Fig 2).[82] The ISI is a measure of the responsiveness of a given thromboplastin to reduction of the vitamin K-dependent coagulation factors compared with the first WHO IRP; the lower the ISI, the more responsive is the reagent. The INR is the PT

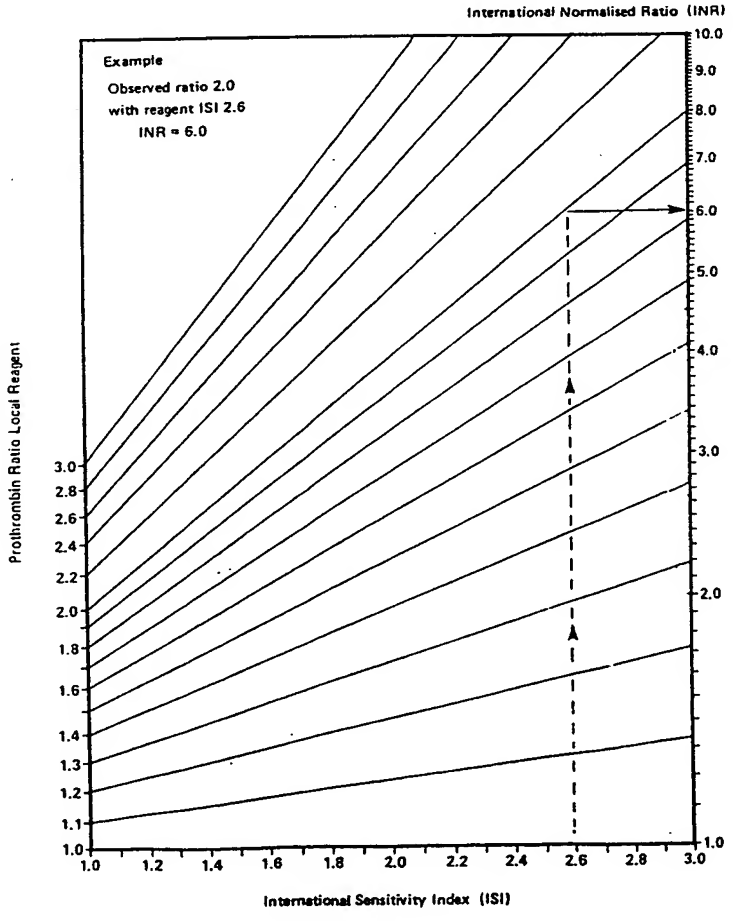


Figure 2. Relationship between the PT ratio and INR over a range of ISI values. At an ISI value of 0.1, the PT ratio is identical to the INR. As the ISI value of the thromboplastin increases, the INR for a given PT ratio also increases.

ratio one would have obtained if the WHO first IRP had been used to perform the PT on the same sample with the manual PT technique.[69,80]

Most commercial manufacturers are now providing ISI values for their reagents and the INR system of PT standardization is being adopted by an increasing number of hospitals in North America. Recently, thromboplastins with recombinant tissue factor have been introduced. They have an ISI of approximately 1.0 and therefore the recorded PT ratio is essentially equivalent to the INR. The increased implementation of INRs in the United States is reflected by data from the College of American Pathologists Comprehensive Coagulation Survey that shows that participant usage climbed from 21.1% in 1991 to 97.3% in 1997.[83]

With the increasing use of the INR system to replace the PT ratio method of reporting, a number of problems have been identified with the INR system. These problems are listed in Table 4.

Potential Problems With the INR System

1. The Lack of Reliability of the INR System When Used at the Onset of Warfarin Therapy and for Screening for a Coagulopathy in Patients With Liver Disease

The PT is responsive to reduction of three of the four vitamin K-dependent procoagulants, factor II, factor VII, and factor X, but individual thromboplastin reagents vary in their sensitivity to decreases in these clotting factors,[84,85] particularly to factors VII and X. Since these three vitamin K-dependent clotting factors have varying rates of plasma clearance, their relative contributions to the prolongation of the PT are different during the induction phase of warfarin therapy (first few days) than during the subsequent weeks to months of treatment.[86] Thus, during the first 2 to 5 days of warfarin treatment, the prolongation of the PT is mainly the result of a reduction in the level of functional factor VII, with some contribution from a decrease in factor X levels. In contrast, during longer-term anticoagulation, the prolongation of the PT reflects a decrease in all three of the vitamin K-dependent coagulation factors.

Table 4—*Potential Problems With the INR*

Problems
1. Lack of reliability of the INR system when used at the onset of warfarin therapy and for screening for a coagulopathy in patients with liver disease.
2. Relationship between precision of the INR determination and reagent ISI.
3. Effect of instrumentation in ISI values.
4. Lack of reliability of the ISI result provided by the manufacturer.
5. Incorrect calculation of the INR resulting from the use of inappropriate control plasma.
6. Problems with citrate concentration and interference with lupus anticoagulant with thromboplastins with low ISI values.

The INR system is based on ISI values derived from the plasma of patients stabilized on anticoagulant treatment for at least 6 weeks.[87] As a result, the INR is less reliable early in the course of warfarin therapy, particularly if results are obtained from different laboratories. However, even in this situation, the INR system is much more reliable than the PT in seconds or the unconverted PT ratio and, from the point of view of clinical management, it appears to be an effective and safe way of monitoring warfarin therapy.[88] Therefore, we recommend that the INR be used as the method of reporting during initiation and maintenance of warfarin treatment. Use of a single laboratory, particularly during the initiation phase of anticoagulation, may improve the reliability of monitoring.

The ACCP committee were unanimous in their view that the INR system of reporting should be used both during the initiation and maintenance of warfarin treatment.

The reliability of reporting the PT ratio as an INR when screening patients with liver disease has also been questioned. However, the results of a recent publication indicate that the INR is valid in patients with liver disease.[89]

2. Relationship Between Precision of the INR Determination and Reagent ISI

As the INR is calculated by raising the PT ratio to the power of the ISI ($INR = [PT\ ratio]^{ISI}$), it follows that the coefficient of variation (CV) of the INR is related to both the CV of the PT ratio and the value of the ISI. Indeed, if the ISI is assumed to be a constant, then the following formula can be derived: CV of the INR = ISI × CV of the PT ratio.[90] This relationship suggests that the precision of the INR can be improved by using reagents with very low ISIs. Interlaboratory proficiency surveys have provided some evidence for this, but the effect on precision appears to be modest (Fig 3).[83,90–92] A major reason for this is the observation that the CV of the PT ratio is not independent of the ISI. Specifically, more responsive reagents yield longer PTs for a given degree of anticoagulation. Often these longer PTs are associated with poorer precision; that is, the longer the PT, the higher the CV of the PT ratio.[83] Consequently, as the ISI decreases, the CV of the PT ratio increases. Optimal precision is therefore obtained when the product of the ISI and the CV of the PT ratio is lowest. This point has not been defined for most PT test systems in clinical use.

Another contributing factor to interlaboratory imprecision is the observation that the ISI is not a constant—that is, there is error in the ISI value. Variation in the ISI itself contributes to the overall interlaboratory CV of the INR. Recent studies have shown that there is a relationship between the ISI value and its interlaboratory variability, with higher ISI reagents giving a higher interlaboratory CV for the ISI.[93,94] This imprecision of the ISI value may contribute to the imprecision associated with higher ISI reagents on interlaboratory proficiency surveys.

The relationship between precision of the INR and test systems is complex and appears to be dependent on both the reagent and instrument. In general, laboratories

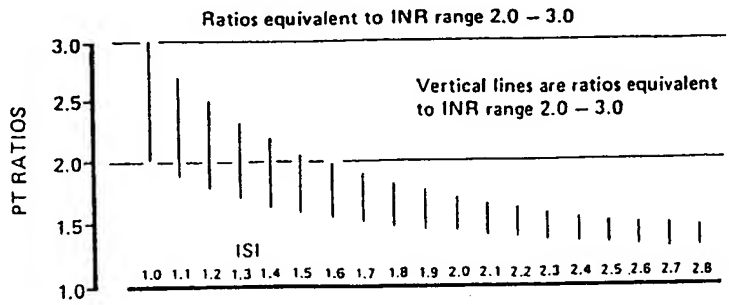


Figure 3. PT ratios equivalent to INR of 2.0 to 3.0 over a range of ISI values.

should select test systems that improve overall INR precision. Use of responsive (ISI < 1.7) reagents with accurate and precise ISI values may significantly improve INR precision. Use of reagents on instruments for which the ISI has not been determined should be avoided, if at all possible.

3. *Effect of Instrumentation on ISI Values*

The INR system is based on a mathematical relationship between the PT ratios obtained with test thromboplastin and the IRP using a manual method of clot detection. However, most laboratories now use automated clot detectors, thereby introducing a new variable. This variable does affect the accuracy of the INR system.[95–100] The variance in the ISI determinations is reduced significantly by calibrating the instrument with lyophilized plasma calibrants prepared by depleting plasma of vitamin K-dependent clotting factors.[94,101,102] The effect can be offset by calibrating each new batch of thromboplastin reagent with plasmas with certified PT values.[94,101,102] Thus, the problem resulting from the use of automated instruments to measure the INR can be minimized by using sensitive thromboplastins (ISI values of < 1.7) and by calibrating each new batch of thromboplastin with plasma calibrants with certified PT values. A similar conclusion was reached by a consensus of the College of American Pathologists further stating that laboratories should use reagent/instrument combinations for which the ISI has been established.[103]

4. *Lack of Reliability of the ISI Result Provided by the Manufacturer*

A number of investigators have noted that the ISI value provided by the manufacturer for each new batch of thromboplastin reagent may be incorrect.[104–106] This problem can be solved by ensuring that manufacturers assign reliable ISI values to their thromboplastins. Commercial reagents should be calibrated against the relevant IRP or in-house standard in a full fresh plasma calibration. Local calibrations can be performed with plasma samples with known certified PT values to determine the instrument-specific ISI.

5. *Incorrect Calculation of the INR Resulting From the Use of Inappropriate Control Plasma*

The PT ratio is calculated by dividing the patient's PT by the mean normal plasma PT. The mean normal plasma PT is not interchangeable with a laboratory control PT, since these values can be substantially different.[107] Therefore, the use of a laboratory control PT instead of a properly defined mean normal PT can lead to erroneous INR calculations, particularly with less responsive reagents. The mean normal PT is determined by measuring the PT on fresh plasma samples obtained from at least 20 healthy individuals of both sexes over a range of age groups. Since the distribution of PT values is not normal, log-transformation and calculation of a geometric mean are recommended. The mean normal PT should be determined with each new batch of thromboplastin reagent using the same instrument as is used to assay the patient's PT.[107]

6. *Problems With Citrate Concentration and Interference With Lupus Anticoagulant With Thromboplastins With Low ISI Values*

The concentration of citrate used to anticoagulate plasma does have an effect on the INR obtained.[108–110] In general, higher citrate concentrations (3.8%) lead to higher INR values; in one recent study, the INR was an average of 19% greater with 3.8% citrate.[108] In addition, with the higher citrate concentration, it is more likely that minor underfilling of the collection tube will cause spurious prolongation of the PT due to the excess citrate present in the test system under such conditions. The ISI of reagents is generally calibrated with plasmas collected into 3.2% citrate while about two thirds of laboratories in the United States use 3.8% citrate as an anticoagulant.[83] In some cases, laboratories may receive samples collected into either 3.2% or 3.8% citrate. Often the concentration used to collect the sample is not known by the laboratory. This creates a problem for the laboratory, as it cannot apply a uniform correction factor for the effect of citrate. For all these reasons, there is an emerging consensus that laboratories should use 3.2% citrate for all routine coagulation studies. However, use of 3.8% citrate is still recommended for platelet aggregation studies.

Lupus anticoagulants are known to be associated with an increased risk of thrombosis. Consequently, it is not uncommon for patients with lupus anticoagulants to be placed on a regimen of oral anticoagulant therapy. Evidence form observational studies suggests that the therapeutic range for patients with lupus anticoagulants is closer to 2.5 to 3.5 rather than 2.0 to 3.0.[111,112] The reasons why a higher INR is necessary are not known. It is possible that the requirement for a higher INR is due to lupus

anticoagulants interfering with the PT. Lupus anticoagulants typically cause prolongation of the activated partial thromboplastin time, but they may also cause mild prolongation of the PT or, in the presence of specific antibodies to prothrombin, significant prolongation of the PT. The degree of prolongation of the PT induced by lupus anticoagulants appears to be dependent on the reagent used.[113] In a recent study, it was found that the INR obtained on samples from patients with lupus anticoagulants receiving oral anticoagulants differed significantly between reagents. The difference in INR values between reagents ranged from 0.4 to 6.5.[113] Della Valle et al[114] also found a discordance between INRs obtained with different reagents on samples from patients with lupus anticoagulants receiving oral anticoagulant therapy. These data point to a synergistic effect on the PT by lupus anticoagulants and oral anticoagulant therapy.

The optimal method of monitoring patients with lupus anticoagulants has not been defined using rigorous methods. In addition to modifying the therapeutic range, as suggested by observational studies, it has been suggested that the prothrombin and proconvertin test, in which supplemental bovine fibrinogen and factor V are added, may be a useful assay in patients with lupus anticoagulants.[115,116] In this study, the patient's dose was adjusted to give a prothrombin and proconvertin result of 15 to 20%. A standard PT was then performed at the same time to determine the corresponding therapeutic PT/INR. The patient was subsequently followed up with routine PTs/INRs. Other alternatives include measurement of prothrombin activity or native prothrombin concentration.[78,113,117,118]

Practical Dosing

Following administration of warfarin, an observable anticoagulant effect is delayed until newly synthesized dysfunctional vitamin K-dependent clotting factors replace the normal clotting factors as the latter are cleared from the circulation. Depending on the dose administered, the delay may range from 2 to 7 days. If a rapid anticoagulant effect is required, an initial dose of heparin should be used and overlapped with warfarin for at least 4 days. Although it has been common practice to commence warfarin therapy with a loading dose, such an approach is unnecessary in most patients, and there are theoretical reasons to start with an estimated maintenance dose of about 5 mg of warfarin; this usually results in patients reaching an INR of 2.0 in about 4 or 5 days. Heparin treatment is usually discontinued when the INR has been in the therapeutic range for at least 2 days. If treatment is not urgent (*eg*, chronic stable atrial fibrillation), treatment can be commenced out-of-hospital with an anticipated maintenance dose of 4 to 5 mg/d, which usually achieves a stable anticoagulant effect in about 6 days.[79]

There is room for flexibility in selecting a starting dose of warfarin. Some clinicians prefer to use a larger starting dose (*eg*, 7.5 to 10 mg) and perform daily INRs if there is urgency in obtaining a therapeutic INR more quickly. However, lower starting doses than 5 mg might be appropriate in the elderly and in patients at high risk of bleeding.

PT monitoring is usually performed daily until the therapeutic range has been achieved and maintained for at least 2 consecutive days, then twice or three times weekly for 1 to 2 weeks, then less often, depending on the stability of PT results. If the PT response remains stable, the frequency of testing can be reduced to intervals as long as every 4 weeks. If adjustments to the dose are required, then the cycle of more frequent monitoring is repeated until a stable dose response is again achieved. Some patients receiving long-term warfarin therapy are difficult to manage because they have unexpected fluctuations in dose response. The unexpected fluctuations in dose response could be due to changes in diet, inaccuracy in PT testing, undisclosed drug use, poor patient compliance, surreptitious self-medication, or intermittent alcohol consumption.

Improving Anticoagulant Control

The effectiveness and safety of warfarin are critically dependent on maintaining the INR in the therapeutic range. Therefore, every effort should be made to maintain the patient in the designated therapeutic range. This objective is facilitated by always aiming for an INR level that is in the mid-level of the INR range (*ie*, 2.5 for a designated range of 2.0 to 3.0 and 3.0 for a designated range of 2.5 to 3.5). The impact of maintaining good anticoagulant control was highlighted by reanalysis of the primary prevention trials in atrial fibrillation using an on-treatment analysis.[119] The results of the on-treatment analysis showed that many of the events (both thromboembolic and bleeding) occurred when the PT ratio was outside the designated therapeutic range and that both the safety and efficacy of warfarin were increased by maintaining good anticoagulant control. Subgroup analyses of other cohort studies have also shown a sharp increase in the risk of bleeding when the INR is higher than the upper limit of the therapeutic range.[120–123]

Additional approaches are being developed to improve anticoagulant control. These include (1) anticoagulation management services (anticoagulation clinics) to manage therapy, (2) point-of-care PT testing that allows patient self-testing and patient self-management of dose adjustments, and (3) computer programs to aid in dose adjustment.

Anticoagulation Management Services

In North America, oral anticoagulation therapy is usually managed by a patient's personal physician (routine medical care [RMC]).[124] An alternative approach is the use of anticoagulation management services (AMS) or anticoagulation clinics,[125] in which the management is conducted by registered nurses, nurse practitioners, pharmacists, or physician assistants using dosage-adjustment protocols developed by experts in the field. The latter approach has the advantage of better coordination by health professionals who acquire in-depth experience

Table 5—*Frequency of Major Hemorrhage/Thromboembolism in Patients Managed Under RMC vs AMS*

Source, yr	Model of Care	No. of Patients	No. of Patient Years	Years of Data Collection	Indications	Target* PT/INR	Major Hem,† %	Fatal‡ Hem	Minor Hem,† %	Rec TE,† %
Garabedian-Ruffalo et al,[126] 1985	RMC	26	64.3	1977–1980	Ven & Art§	1.5–2.5	12.4	0	NA	6.2
	AMS	26	41.9	1980–1983	Ven & Art	1.5–2.5 (PTR)	2.4	0	NA	0
Cortelazzo et al,[127] 1993	RMC	271	677	1982–1990	Mechanical valves	25–35%‖	4.7	0	NA	6.6
	AMS	271	669	1987–1990	Mechanical valves	3.0–4.5 (INR)	1.0	0	NA	0.6
Wilt et al,[128] 1995	RMC	44	28	1988–1993	Ven & Art	NA	17.8	0	7.1	42.8
	AMS	68	60	1988–1993	Ven & Art	NA	0	0	3.3	0
Chiquette et al,[129] 1998	AMS	82	199	1977–1986	Ven & Art	NA	1.5	NA		3.5
	RMC	142	102	1991–1992	Ven & Art	2.0–4.5	3.9	1	35.3	11.8
	AMS	176	123	1992–1994	Ven & Art	2.0–4.5 (INR)	1.6	0	8.1	3.3

*Target PTR = PT ratio unless INR indicated.
†Events expressed as per patient-year of therapy. HEM = hemorrhage; TE = thromboembolism.
‡Fatal hemorrhagic events included with major hemorrhage.
§Ven & Art = mixed indications in the venous and arterial systems.
‖Prothrombin activity.

managing large numbers of patients. Unfortunately, most data comparing clinical outcomes achieved with one or the other of these two models of management (RMC vs AMS) are retrospective.

Clinical Outcomes: RMC vs AMS

Table 5 summarizes the four studies in which investigators used clinical outcomes to compare two models of care in a single setting.[126–129] All of these studies used a before and after design and none were prospective randomized trials. In two studies,[126,127] the same patient groups were observed first in an RMC setting and then in an AMS setting. The third study[128] involved two defined cohorts of patients[128] and the fourth report[129] provided data on three sequential inception cohorts: an initial AMS, then an RMC cohort, followed by a second AMS cohort.[129] Although none of these trials were randomized, each reported an impressive reduction in the incidence of major hemorrhage and thromboembolism: and the one study that evaluated death due to bleeding or thromboembolism found a reduction that approached statistical significance ($p = 0.09$).[129]

These results are similar to a number of nonrandomized, observational studies of either RMC[130,131] or an AMS[122,132–142] where observed rates of hemorrhage were 7.6% vs 2.8% per patient-year of therapy, respectively, and observed rates of thrombosis were 8.1% vs 2.6% per patient-year of therapy, respectively.

Although these results suggest that the coordinated approach of anticoagulation clinics is superior to RMC, the studies were not randomized; therefore, they need to be validated.

Cost-Effectiveness of RMC vs AMS

Attempts have been made to compare the relative costs of the two approaches. Gray et al[143] estimated a savings of $860 per patient-year of therapy (in 1985 dollars) due to reduced hospital days in his study of patients treated by an AMS vs RMC. Chiquette et al[129] found a savings of $1,650 per patient-year of therapy in their comparative study due to a significant reduction in hospitalizations and emergency department visits. The fact that neither of the two studies was properly randomized means that the observations may not be valid. Even so, in the latter two periods of the study by Chiquette et al,[129] > 90% of all patients started on a regimen of anticoagulation therapy in the health-care setting were included in the analysis and the two groups were similar in terms of demographics, indications for anticoagulation, and concomitant conditions.

Point-of-Care Patient Self-Testing and Patient Self-Management

Recent technological advances in point-of-care PT measurement offer the potential for both simplifying and improving oral anticoagulation management. Three classes of portable PT monitors, suitable for patient self-testing at home, are currently available for point-of-care diagnostic testing[144] (Table 6). Each of these monitors measures the thromboplastin-mediated clotting time that is then converted to a plasma PT equivalent by a microprocessor and expressed as a PT or INR.

The validity of this method was initially established in 1987 by Lucas et al[145] with an instrument (Coumatrak; Biotrack Inc; Freemont, CA). Using 858 samples from 732 subjects (control subjects, warfarin-treated patients, and heparin-treated patients), Lucas et al[145] reported a correlation coefficient of 0.96 between reference plasma PTs and capillary whole blood PTs. Results were similar for capillary and venous whole blood measured on the point-of-care instrument. Within-day precision using two different levels of controls revealed CVs of 4.9% and 2.9%. The accuracy of the instrument was not compromised by hematocrit measurements ranging from 23 to 54%.

Other studies have confirmed the accuracy of the instrument compared to reference laboratory methods with correlation coefficients of 0.95[146] and 0.91.[147]

Table 6—*Capillary Whole Blood (Point-of-Care) PT Instruments*

Name of Instrument	Method
Protime Monitor 1000*	Thromboplastin based
Coumatrak*	Clot detection: cessation of blood flow through capillary channel
Ciba Corning 512 Coagulation Monitor*	
CoaguChek Plus*	
CoaguChek	Thromboplastin based
Thrombolytic Assessment System	Clot detection: cessation of movement of iron particles
ProTIME Monitor	Thromboplastin based
	Clot detection: cessation of blood flow through capillary channel
Avocet PT 1000†	Thromboplastin based
	Clot detection: fluorescent thrombin substrate

*All instruments based on original Biotrack model (Protime Monitor 1000) and licensed under different names and now marketed as CoaguChek Plus (some with added capabilities).
†Not yet commercially available.

A study by Jennings et al[148] using a coagulation monitor (Ciba Corning Biotrack 512; Medfield, MA)[148] examined 104 patients receiving warfarin and 20 healthy subjects with capillary PTs and compared the results with two standard laboratory methods. They found poor comparability between the instrument PT and the thrombotest. McCurdy and White[149] studied 143 paired specimens and found that the capillary method yielded the most accurate results in an INR range of 2.0 to 3.0, but that the results of the two methods became discrepant as the INR increased. They also assessed precision of two repeated measurements in 54 patients and found a within-patient SD of 0.23 INR units for the capillary whole blood PT and 0.19 INR units for paired clinical laboratory measurements. Tripodi et al,[150] using the 512 Coagulation Monitor (Ciba Corning Diagnostics; Medfield, MA) found that by recalibrating the ISI of the instrument's thromboplastin against the secondary IRP for rabbit thromboplastin, it was systematically higher (ISI, 2.715) than that reported by the manufacturer (ISI, 2.036). Like McCurdy and White,[149] they found that the monitor underestimated the results as the INR increased (INR > 4.0). However, this error was not instrument related but the result of a faulty ISI; the error did not occur when the INR was recalculated using their recalibrated ISI.

Using a second class of PT monitors (CoaguChek; Boehringer Mannheim Corp; Indianapolis, IN) Oberhardt et al[151] compared the instrument with standard laboratory methods and reported a correlation coefficient of 0.96 in 271 samples. Rose et al[152] determined within-day precision for normal and abnormal control plasmas (20 tests each) that yielded CVs of 3.7% and 3.6%, respectively. A correlation coefficient of 0.86 was obtained from 50 outpatients (using capillary whole blood) compared with reference plasma PTs. Fabbrini et al[153] also compared this technology with standard laboratory methods and found reasonable precision (CV = 6% and 4%) with excellent correlation coefficients of 0.92 and 0.91 compared with reference plasma PTs.

Tripodi et al[154] evaluated the calibration of the ISI in this system based on an IRP and found that they were extremely close to those adopted by the manufacturer for both whole blood and plasma. Although the CVs of the slopes of the regression lines comparing the system with an international reference were excellent (CV of 2.2 for both whole blood and plasma on the instrument compared with the international reference), the instrument reported significantly higher INRs (3.20 and 3.41 in whole blood and plasma vs 2.92 for plasma in the reference system) using the manufacturer's calibration. The differences were due to a lower mean normal PT adopted by the manufacturer.

A recent study by Kaatz et al[155] evaluated both classes of monitors (CoaguChek and Biotrack) as well as four clinical laboratories against the criterion standard established by the WHO using an international reference thromboplastin and the manual tilt-tube technique. They found that laboratories using a more sensitive thromboplastin (ISI = 1.99 and 2.0) showed close agreement with the criterion standard, whereas laboratories using an insensitive thromboplastin (ISI = 2.84 and 2.98) showed poor agreement. The two monitors fell between these two extremes. As in the study by McCurdy and White,[149] the Coumatrak underestimated the INR at values above 2.5, whereas the CoaguChek simply showed more scatter at INR values above 2.75. INR determinations of the Coumatrak monitor and the CoaguChek were only slightly less accurate than those of the best clinical laboratories.

A third class of point-of-care capillary whole blood PT instrumentation (ProTIME Monitor; International Technidyne Corporation; Edison, NJ) has been developed. This differs from the previously described instruments in that it performs a PT in triplicate (three capillary channels) and simultaneously performs a level 1 and level 2 control (two additional capillary channels). In a multi-institutional trial,[156] simultaneous capillary whole blood and venous samples from 201 warfarin-treated patients and 52 control subjects were compared with standard laboratory methods at each institution as well as with a reference laboratory. The ProTIME INR correlated well with the reference laboratory for both the health-care provider (venous sample, r = 0.93) and the patient (capillary sample, r = 0.93). PT results for fingersticks performed by both the patient and the health-care provider were equivalent and correlated highly (r = 0.91).

In a separate report in children, Andrew et al[157] reported on the instrument's accuracy and precision in 76 warfarin-treated children and 9 healthy control subjects. Venous and capillary whole blood tested on the instrument yielded a correlation of r = 0.89. Both results, compared with venous blood tested in a reference laboratory (ISI = 1.0), revealed correlation coefficients of 0.90 and 0.92, respectively.

Patient Self-Testing

Anderson et al[158] confirmed the feasibility and assessed the accuracy of patient self-testing at home in a group of 40 individuals who monitored their own therapy over a period of 6 to 24 months. Based on either narrow or expanded target therapeutic ranges, they observed a mean level of agreement per patient with reference plasma PTs of 83% by narrow criteria and 96% by expanded criteria. Ninety-seven percent of the patients preferred home testing to standard management.

White et al,[159] in a small randomized study, assessed patients' ability to measure their own PT following hospital discharge with warfarin dosing managed by their health-care providers. These self-testing patients (n = 23), compared with a control group treated by an AMS (n = 23), spent a greater percentage of the time in therapeutic range (93% vs 75%; p = 0.003) and were significantly less likely to be in the subtherapeutic range during the follow-up period (6.3% vs 23%; p < 0.001). This study was underpowered to detect differences in outcomes of hemorrhage or thrombosis.

In a larger randomized study published in abstract form, Beyth and Landefeld[160] reported on 325 newly treated elderly patients, 163 of whom had their dose managed by a single investigator based on INR results from patient self-testing at home compared with 162 treated by their private physicians based on venous sampling. Over a 6-month period, the investigators recorded a rate of major hemorrhage of 12% in the latter group vs 5.7% in the self-testing group. This finding was based on an intention-to-treat analysis. For those actually performing self-testing, there was only a 1.2% incidence of major hemorrhage.

Patient Self-Management

In 1974, Erdman et al[161] first tested the concept of patient self-management of oral anticoagulation based on physician-derived guidelines with PTs obtained on plasma samples by routine laboratory instrumentation. In nearly 200 patients with prosthetic heart valves managing their own therapy, they claimed a greater degree of satisfactory anticoagulation (98% of 195 patients enrolled) compared with a retrospective survey of standard management patients who achieved only a 71% degree of adequate anticoagulation.

Ansell et al[162,163] analyzed the results of patient self-management with the Biotrack instrument over a span of 7 years in a cohort of 20 patients ranging in age from 3 to 87 years with diverse indications for anticoagulation. These patients performed their own PT test at home using the Biotrack instrument and adjusted their own warfarin dose based on physician guidelines. The results were compared with an age-, sex-, and diagnosis-matched control group treated by an AMS. Self-managed patients were found to be in therapeutic range for 88.6% of the PT determinations compared with 68% for the control subjects (p < 0.001). There were also fewer dose changes for study patients (10.7%) than for control subjects (28.2%; p < 0.001), while complication rates did not differ between the groups. Patient satisfaction was extremely high with this mode of therapy based on a patient survey of attitudes.

In a retrospective study, Bernardo[164] reported on 216 self-managed patients between 1986 and 1992, and found 83.1% of the PT results were within target therapeutic range and no serious adverse events occurred. The results in a randomly selected subgroup of 92 self-managed patients were compared with a historical cohort of 317 patients managed by traditional means (118 patient-years vs 374 patient-years); there was a trend for fewer hemorrhagic events (3.38% vs 4.38%) and fewer embolic events in the self-managed group.

Most recently, Horstkotte et al[165] published in abstract form the outcomes from a randomized prospective study of 150 patients with prosthetic heart valves who managed their own therapy (n = 75) compared with a control group (n = 75) who were managed by their private physicians (RMC). The self-managed patients tested themselves approximately every 4 days and achieved a 92% degree of satisfactory anticoagulation as determined by the INR. The physician-managed patients were tested approximately every 19 days and only 59% of INRs were in therapeutic range. The self-managed individuals experienced a 4.5%/y incidence of any type of bleeding and a 0.9%/y rate of thromboembolism compared with a 10.9% and 3.6% rate, respectively, in the physician-managed group (p = 0.038 between the two groups).

Summary and Conclusions

Based on a number of observational studies, patients treated by an AMS achieve better therapeutic outcomes than those treated under a model of RMC. This difference may be due to maintaining better therapeutic control in the AMS, but it could be due to bias. Better-quality studies are needed to confirm these differences.

Newer models of warfarin management are emerging based on point-of-care PT testing, allowing patients to determine their own PT and adjust their own warfarin dose. Initial results of studies suggest that this model of dose management may result in better clinical outcomes when compared with RMC, but randomized controlled trials comparing patient self-management with management by an AMS are needed before recommendations can be made about the value of this model of care.

Computerized Systems for Predicting Warfarin Dosage

A number of computerized programs have been developed to assist physicians and other health-care providers with oral anticoagulant dosing.[166–169] In a

recent randomized trial, the reliability of three established computerized dosage programs was compared with warfarin dosing by experienced medical staff in patients who attended the same established outpatient clinic.[170] All three programs gave satisfactory control compared with empiric dosage adjustment by experienced physicians in the 2.0 to 3.0 INR target range. In patients requiring more intense therapy (3.0 to 4.5 INR), the computerized programs achieved significantly better control than empiric dosage adjustment. Ageno and Turpie[171] completed a randomized, prospective study in 101 long-term anticoagulated patients with prosthetic cardiac valves comparing a computerized system with standard manual management by trained personnel. The computer program was comparable to the manual system in maintaining the percentage of INRs in range, but achieved a 50% reduction in the number of dose adjustments. More recently, a large multicenter randomized study of 285 patients performed by the European Concerted Action on Anticoagulation[172] showed that a computer-assisted dosage program was significantly more effective than traditional dosing in achieving a targeted therapeutic range. Thus, computerized dosage adjustments appear to have an advantage over traditional dosage adjustments.

Management of Patients With High INR Values With or Without Bleeding

If a patient has an elevated INR and is not bleeding or does not require surgery, then it is reasonable to reduce the INR to a safer level of < 5.0 either by omitting a dose of warfarin or by administering vitamin K_1. If the patient has serious bleeding, the INR should be reduced to 1.0 as soon as possible. If the patient requires elective or urgent surgery, it is reasonable to reduce the INR to 1.0 to 1.5 at the time of surgery.

Three approaches can be used to reduce the INR. The first and least rapid is to discontinue treatment, the second is to use vitamin K_1, and the third and most rapid is to transfuse the patient with fresh plasma or prothrombin concentrate. The choice of these approaches is based largely on clinical judgment since randomized trials using clinical end points have not been performed (to our knowledge).

Discontinuation of Warfarin Therapy

White and associates[159] reported that it takes about 4 days for the INR to return to the normal range when warfarin therapy is stopped in patients whose INR is between 2.0 and 3.0.

Vitamin K_1

Ideally, vitamin K_1 should be administered in a dosage that rapidly reduces the INR into a safe range without (1) overshooting the lower limit of the targeted range, (2) rendering the patient resistant to warfarin when therapy with it is restarted, and (3) exposing the patient to a risk of an anaphylactoid reaction. There is a strong relationship between the level of the INR and the risk of bleeding. The risk of bleeding rises sharply when the INR exceeds 5.0, but bleeding is increased, particularly in high-risk patients, when the INR exceeds 3.0.

To our knowledge, the optimal dosage and the optimal route of administration of vitamin K_1 have never been compared using a rigorous study design. Consequently, there is no uniformly accepted approach. Recommendations vary from doses ranging from < 1 mg to up to 10 mg and all three routes of administration—IV, subcutaneous, and oral—have been advocated.

High doses of vitamin K_1 are effective but can lead to warfarin resistance for up to a week after they are discontinued. The IV route is likely to give the most predictable response, but it can be complicated by anaphylactoid reactions, and there is no definitive evidence that this serious, but rare, complication can be avoided by using low doses IV. For these reasons, there has been recent interest in evaluating low doses of vitamin K_1 (to avoid overcorrection and warfarin resistance) administered by the oral or subcutaneous routes (to avoid anaphylactoid reactions). In the past issues of the *Proceedings of the ACCP Consensus Conference,* subcutaneous vitamin K_1 was advocated for nonemergency situations and IV vitamin K for emergency situations. However, one recent report suggested that the response to subcutaneously administered vitamin K may be delayed and unpredictable,[173] while other new studies have confirmed older reports that the oral route is effective, and it has the advantages of being safer and more convenient than parenteral administration.

Over 20 years ago, a number of descriptive studies reported that the anticoagulant effect of warfarin can be reversed by oral vitamin K_1.[174–176] Interest in the use of oral vitamin K_1 to reverse the anticoagulant effect of warfarin waned, largely because of a perception that the response to oral vitamin K_1 is variable. However, as pointed out by Weibert and associates,[177] this perception is based largely on a study that measured plasma concentrations of vitamin K_1 rather than the PT.[178] The early observations of the effectiveness of vitamin K_1 were confirmed by a randomized trial by Pengo and associates[179] in 1993 who demonstrated that 2.5 mg of oral vitamin K_1 was more effective than withholding warfarin at correcting the INR to < 5.0 at 24 h. More recently, a number of cohort studies have been performed to identify an appropriate dose of oral vitamin K_1.

Crowther and associates[180] performed a prospective cohort study in 62 patients who were treated with warfarin and had INR values between 4.0 and 10.0. None of the patients had bleeding complications. The next dose of warfarin was omitted and 1.0 mg of oral vitamin K_1 was administered. The INR was lowered at 24 h in 59 of the 62 patients (95%); it was reduced to < 4.0 in 53 (85%), to between 1.9 and 4.0 in 50%, and to < 1.9 in 22 patients (35%). None of the patients showed resistance to warfarin when oral anticoagulant therapy was recommenced. The results of this study suggest that in patients with moderately elevated INR values (4.0 to 10.0), 1 mg of oral vitamin K_1 is effective for reversing an excessive anticoagulant effect of warfarin within 24 h.

In a retrospective cohort study, Weibert and associ-

ates[177] evaluated the effectiveness of 2.5 mg of oral vitamin K_1 for reversing an excessive warfarin effect in 81 patients with an INR of 5.0 to > 10.0. Ninety percent of the patients achieved an INR of < 5.0 and only 17% developed an INR of < 2.0. An INR of < 5.0 was achieved in 48 h in all patients whose initial INR was < 9.0. However, a dose of 2.5 mg of oral vitamin K_1 failed to lower the INR to < 5.0 in five of eight patients (63%) whose initial INR was > 9.0.

The results of these studies indicate that a low dose oral vitamin K_1 is effective for reducing the INR in patients treated with warfarin. A dose of 1.0 to 2.5 mg is effective when the initial INR is between 5.0 and 9.0, but (based on the results of the study reported by Weibert and associates[177]), a larger oral dose, *eg*, 5 mg, is required when the INR is > 9.0.

Since oral vitamin K_1 is effective for lowering the INR, it is the route of choice unless rapid reversal of the INR is considered to be critical, in which case vitamin K_1 can be administered by slow IV infusion.

Recommendations

Since none of these studies used clinical end points to assess outcomes, all recommendations are grade C2:

(1) If the INR is above the therapeutic range but below 5.0, the patient does not have a clinically significant bleeding, and rapid reversal is not indicated for reasons of surgical intervention, the dose can be lowered or the next dose can be omitted and warfarin therapy can be resumed at a lower dose when the INR approaches the desired range. If the INR is only minimally above the therapeutic range, then no dose reduction may be required at all.

(2) If the INR is above 5.0 but below 9.0 and the patient does not have clinically significant bleeding, then one of two approaches can be used. If the patient has no additional risk factors for bleeding, the next one or two doses of warfarin can be omitted, the INR monitored more frequently, and warfarin therapy reinstituted at a lower dose when the INR falls into the therapeutic range. Alternatively, the next dose of warfarin should be omitted and vitamin K_1 (1 to 2.5 mg) can be given orally. This second approach should be used if the patient is at increased risk of bleeding.

When more rapid reversal is required because the patient requires urgent surgery or dental extraction, then vitamin K_1 can be given orally in a dose of 2 to 4 mg with the expectation that a reduction of the INR will occur within 24 h. If the INR remains high at 24 h, an additional dose of 1 or 2 mg of vitamin K_1 can be given.

(3) If the INR is above 9.0 and the patient does not have clinically significant bleeding, a higher dose of vitamin K_1 (3 to 5 mg) should be given orally with the expectation that the INR will be reduced substantially by about 24 to 48 h. The INR should be monitored closely and treatment with vitamin K_1 can then be repeated, if necessary, in a similar manner.

(4) If a very rapid reversal of an anticoagulant effect is required because of serious bleeding or major warfarin overdose (*eg*, INR > 20.0), vitamin K_1 in a dose of 10 mg should be given by slow IV infusion and supplemented with fresh plasma transfusion or prothrombin complex concentrate depending on the urgency of the situation. The administration of vitamin K_1 injection may have to be repeated every 12 h.

(5) In case of life-threatening bleeding or serious warfarin overdose, replacement with prothrombin complex concentrate is indicated, supplemented with 10 mg of vitamin K_1 by slow IV infusion; this can be repeated if necessary depending on the INR.

(6) If continuing warfarin therapy is indicated after high doses of vitamin K_1 administration, then heparin can be given until the effects of vitamin K_1 have been reversed and the patient becomes responsive to warfarin therapy.

Management of the Patient Receiving Long-Term Warfarin Therapy Who Requires Surgery

This subject has been reviewed recently.[181] Several approaches can be used to manage patients who are being treated with warfarin for an underlying thrombotic disorder and require surgery. The choice depends on personal preferences and the risk of thrombosis. With each of the following options, the length of time for warfarin dosage reduction and for heparin or low-molecular-weight heparin (LMWH) use preoperatively can be shortened by administering vitamin K_1 24 to 48 h before surgery to reverse the warfarin and beginning heparin at that time.

The first approach is to stop warfarin therapy 4 to 5 days before surgery and use postoperative prophylaxis with a combination of low-dose heparin therapy, 5,000 U subcutaneously, and warfarin therapy.

The second is to stop warfarin therapy 4 to 5 days before surgery and replace it with low-dose heparin therapy, 5,000 U subcutaneously preoperatively, or a prophylactic dose of LMWH and then commence low-dose heparin (or LMWH) and warfarin therapy postoperatively.

The third is to stop warfarin therapy 4 to 5 days before surgery and replace it with full-dose heparin or full-dose LMWH therapy. Therapy can be administered by subcutaneous injection as an outpatient; it can then be given as a continuous IV infusion when the patient is admitted to hospital in preparation for surgery and discontinued 5 h before surgery with the expectation that the anticoagulant effect will have worn off at the time of surgery. It is also possible to continue with subcutaneous heparin or LMWH (without switching to the continuous IV route) and to stop subcutaneous therapy 24 h before surgery with the expectation that the anticoagulant effect will have worn off at the time of surgery.

The fourth approach is to continue with warfarin therapy at a lower dose and operate at an INR of 1.3 to 1.5, an intensity that has been shown to be safe in randomized trials of gynecologic and orthopedic surgical patients. The dose of warfarin can be lowered 4 or 5 days before surgery. Warfarin therapy can then be restarted postoperatively, supplemented with low-dose heparin (5,000 U subcutaneously), if necessary.

A fifth option is available for patients requiring dental procedures. Thus, tranexamic acid or epsilon amino caproic acid mouthwash has been used successfully without interrupting anticoagulant therapy.[182,183]

Management of the Patient Who Bleeds During Warfarin Therapy

The short-term management of patients who bleed with an excessively prolonged INR has been discussed above. The long-term management of patients who bleed but who require protection against systemic embolism (*eg*, patients with mechanical heart valves or with atrial fibrillation and other risk factors) is problematic. There are two general principles that should be followed: (1) to attempt to reverse the cause of bleeding; and (2) to examine the possibility of lowering the intensity of the anticoagulant effect. Every effort should be made to treat the cause of bleeding (*eg*, the use of aggressive antiulcer therapy) if it is potentially reversible.

The risk of bleeding is strongly related to the intensity of the anticoagulant effect. Therefore, every effort should be made to maintain the INR at the lower limit of the therapeutic range (*ie*, 2.0). Laboratory control of treatment should be optimized with frequent INR measurements and by ensuring that a sensitive thromboplastin (ISI about 1.2) is used. For patients with mechanical prosthetic valves (and a persisting risk of increased bleeding), it would be reasonable to aim for an INR of 2.0 to 2.5. For patients with atrial fibrillation (and a persisting risk of increased bleeding), the anticoagulant intensity can be reduced to an INR of 1.5 to 2.0 with the expectation that efficacy will be reduced but not abolished.[2,184] Alternatively, aspirin can be used to replace warfarin in patients with atrial fibrillation.

Clinical Results

The clinical effectiveness of oral anticoagulants has been established for a variety of indications based on the results of well-designed clinical trials. Some of these trials have compared two levels of anticoagulant intensity and have shown that the moderate intensity regimen (INR of 2.0 to 3.0) is as effective, but produces significantly less bleeding, than the more intense regimen (INR of 3.0 to 4.5) for each of the indications in which comparisons were performed (Table 7) (see below).

Oral anticoagulants have been shown to be effective in the following ways: in the primary and secondary prevention of venous thromboembolism; in the prevention of systemic embolism in patients with tissue and mechanical prosthetic heart valves or with atrial fibrillation; in the prevention of acute myocardial infarction in patients with peripheral arterial disease; in the prevention of stroke, recurrent infarction, and death in patients with acute myocardial infarction; and in the prevention of myocardial infarction in men at high risk.[2] Oral anticoagulants are indicated in certain patients with mitral stenosis to prevent systemic embolism, although to our knowledge, their effectiveness has never been demonstrated by a randomized clinical trial. For most indications, a moderate anticoagulant effect with a targeted INR of 2.0 to 3.0 (less intense regimen) is appropriate (Table 7).

The role of anticoagulants in cerebral ischemia of arterial origin is unclear, although it is now clear from the results of the Stroke Prevention in Reversible Ischemia Trial (SPIRIT) study that high-intensity oral anticoagulation (INR, 3.0 to 4.5) is dangerous in these patients.[123] In SPIRIT, study patients with a transient ischemic attack or minor ischemic stroke were randomly assigned to a regimen of oral anticoagulants (INR, 3.0 to 4.5) or aspirin, 30 mg/d. The primary measure of outcome was the composite event "death from all vascular causes, nonfatal stroke, nonfatal myocardial infarction, or nonfatal major bleeding complication." The trial was stopped at the first interim analysis at which time 1,316 patients had been included with a mean follow-up of 14 months. There was an excess of the primary outcome event in the anticoagulated group (81 of 651) vs 36 of 665 in the aspirin group (hazard ratio,

Table 7—*Relationship Between Bleeding and Intensity of Anticoagulant Therapy*

Source, yr	No. of Patients	Anticoagulant Duration	Therapeutic Range (INR)	Total % of Bleeding	p Value
Hull et al,[206] 1982 Deep vein thrombosis	96	3 mo	3.0–4.5 vs 2.0–2.5	22.4 vs 4.3	0.015
Turpie et al,[221] 1988 Prosthetic heart valves (tissue)	210	3 mo	2.5–4.0 vs 2.0–2.5	13.9 vs 5.9	< 0.002
Saour et al,[222] 1990 Prosthetic heart valves (mechanical)	247	3.47 yr	7.4–10.8 vs 1.9–3.6	42.4 vs 21.3	< 0.002
Altman et al,[223] 1991* Prosthetic heart valves (mechanical)	99	11.2 mo	3.0–4.5 vs 2.0–2.9	24.0 vs 6.0	< 0.02

*Patients also given aspirin, 300 mg, and dipyridamole, 75 mg bid.

Table 8—*Oral Anticoagulants vs Aspirin in Transient, Ischemic Attack and Minor Stroke: The SPIRIT Study Group*[123]*

Event	Anticoagulant (INR 3.0 to 4.5)	Aspirin (30 mg)	OR
No./patient-yrs of observation	651/735	665/775	
Vascular death, stroke, MI, major bleeding	81	36	2.3
Death	35	15	2.4
Vascular death	24	11	2.3
Major bleeding	53	6	9.3
All major ischemic events	27	27	1

*OR = odds ratio; MI = myocardial infarction.

2.3; 95% confidence interval, 1.6 to 3.5). This excess was the result of 53 major bleeding complications (27 intracranial; 17 fatal) during anticoagulant therapy vs 6 with aspirin (3 intracranial; 1 fatal). The incidence of bleeding was found to be increased by a factor of 1.43 (95% confidence interval, 0.96 to 2.13) for each 0.5-U increase of the achieved INR. The authors concluded that oral anticoagulants are not safe when used at a targeted INR range of 3.0 to 4.5 in patients after cerebral ischemia of presumed arterial origin (Tables 8 and 9).

Prevention of Venous Thromboembolism

Oral anticoagulants are effective in preventing venous thrombosis after hip surgery[185–187] and major gynecologic surgery[188,189] when used at a targeted INR of 2.0 to 3.0. Benefit has been demonstrated when treatment is commenced a number of days before surgery,[185,186] the evening before surgery, or on the first postoperative day.[187] The risk of clinically important bleeding with the moderate-intensity regimen is small, but because warfarin prophylaxis is more complicated to use than fixed low-dose heparin, warfarin is generally reserved for very-high-risk patients such as those with previous venous thrombosis or those having major orthopedic procedures. For patients undergoing elective hip replacement or major knee surgery, treatment with LMWHs, commenced 12 to 24 h postoperatively, has been shown to be more effective than warfarin therapy commenced postoperatively or the night before surgery.[190,191] Very low fixed doses of warfarin (1 mg daily) have been reported to be effective in one small study in patients having gynecologic surgery[189] and in a larger study in which 1 mg of warfarin per day was effective in preventing subclavian vein thrombosis in cancer patients with indwelling subclavian catheters.[192] Surprisingly, the very-low-dose warfarin regimen was associated with an increase in fibrinolysis in the gynecologic patients.[193] Four recent randomized studies demonstrated that warfarin in a fixed dose of 1 mg is ineffective in preventing postoperative venous thrombosis in patients having major orthopedic surgery.[194–197] Although attractive because of its safety and simplicity, it is now clear that the 1-mg dose is ineffective in hip and knee replacements and that this dose should not be used for these high-risk conditions. Recently, Levine and associates[198] reported that warfarin, 1 mg daily, for 6 weeks followed by low-dose warfarin with a targeted INR of 1.5 is effective in preventing thrombosis in patients at high risk with stage IV breast cancer given chemotherapy.

Table 9—*Oral Anticoagulants vs Aspirin in Transient Ischemic Attack and Minor Stroke Bleeding Complications: The SPIRIT Study Group*[123]

Major Bleeding Complications	Anticoagulant (INR 3.0 to 4.5)	Aspirin (30 mg)
No. of patients	651	663
Fatal intracranial	15	1
Fatal other	2	0
Nonfatal intracranial	12	2
Nonfatal other	24	3
Total	53	6

Treatment of Deep Vein Thrombosis

The optimal duration of oral anticoagulant therapy has been reviewed by Hirsh.[199] The duration of treatment is influenced by the following factors: (1) idiopathic vs secondary thrombosis with a reversible cause—a longer course of therapy should be used if the thrombosis is idiopathic; (2) proximal vs calf vein thrombosis—a longer course of therapy is indicated in patients with proximal vein thrombosis; (3) first episode vs recurrent thrombosis—a longer course of treatment is indicated in patients with recurrent venous thrombosis; and (4) the presence or absence of laboratory evidence of thrombophilia—a longer course of treatment is indicated in patients with thrombophilia.

Oral anticoagulant therapy is indicated for at least 3 months in patients with proximal vein thrombosis,[200,201] for at least 6 months in patients with idiopathic proximal vein thrombosis, for at least 6 months in patients with recurrent venous thrombosis, and for 6 weeks to 3 months in patients with symptomatic calf vein thrombosis.[202–205] Indefinite anticoagulant therapy is indicated in patients with more than one episode of idiopathic proximal vein thrombosis and a recognized thrombophilic defect.

A moderate-dose regimen (INR, 2.0 to 3.0) is as effective as the more intense regimen (INR, 3.0 to 4.5), but it is associated with a much lower incidence of bleeding[206] (Table 7). Recent studies in patients with proximal vein thrombosis evaluating short-course vs long-course heparin treatment[207,208] have confirmed the initial observation that moderate-dose warfarin therapy is associated with a low rate of recurrent venous thromboembolism and a low incidence of bleeding and that 6 months is more effective than 6 weeks of treatment.[203]

Primary Prevention of Myocardial Ischemia

The Thrombosis Prevention Trial[2] evaluated low-intensity warfarin therapy and low-dose aspirin therapy in 5,499 men between the ages of 45 and 69 years at high risk of ischemic heart disease. The primary outcome was the prevention of acute ischemic coronary events defined as

the composite of coronary death and fatal and nonfatal myocardial infarction. The targeted INR was 1.3 to 1.8 and the mean warfarin dose was 4.1 mg/d.

The annual incidence of acute ischemic coronary events was 1.4% per year in the placebo group. Both warfarin and aspirin produced a similar (nonsignificant) reduction in acute ischemic events of 22% and 23%, respectively, while the combination of warfarin and aspirin produced a significant reduction ($p = 0.006$) of 34%. Although more effective, combined treatment was associated with an increased risk of hemorrhagic stroke.

The results are important because they show (1) that a targeted INR of 1.3 to 1.8 is effective in preventing acute ischemic events, particularly fatal events, and (2) that the combination of low-intensity warfarin therapy and low-dose aspirin therapy is more effective than either agent used alone, but that the combination increases the risk of bleeding.

On superficial examination, the effectiveness of low-intensity warfarin therapy in the thrombosis prevention study appears to be at odds with the results of the Coumadin Aspirin Reinfarction Study study[209] and the Stroke Prevention in Atrial Fibrillation (SPAF) III study.[210] However, in both of these latter studies, fixed-dose warfarin therapy was used, while in the Thrombosis Prevention Trial,[2] the warfarin dose was adjusted and varied over a wide range (from 0.5 to 12.5 mg/d).

Acute Myocardial Infarction

There is evidence from studies performed in the 1960s that moderate-dose warfarin therapy (INR, 2.0 to 3.0) is effective in preventing stroke and venous thromboembolism in patients with acute myocardial infarction (AMI). More recently, three studies reported that high-intensity anticoagulant therapy (INR of approximately 3.0 to 4.5) is effective in reducing recurrent infarction, stroke, and death.

The early evidence that oral anticoagulants are effective for the early treatment of AMI comes from studies performed in the 1960s and 1970s, which reported that a moderate-intensity warfarin regimen (presumptive INR, 1.5 to 2.5) is effective for preventing stroke and pulmonary embolism.[211–215] Three randomized trials evaluated the effectiveness of oral anticoagulants in patients with AMI;[211–213] two of these, the Medical Research Council study and the Veterans Affairs cooperative study, showed a significant reduction in stroke[211,213] and the third, the Bronx Municipal Study, reported a nonsignificant trend.[212] One of the studies, the Bronx Municipal Study,[212] showed a significant reduction in mortality, while the other two[211,213] showed no effect on mortality. There was also a reduction in the incidence of clinically diagnosed pulmonary embolism in all three studies.[211–213]

The early evidence that oral anticoagulants are effective in the long-term management of AMI comes from analysis of pooled data from seven randomized trials published between 1964 and 1980 which showed that oral anticoagulant therapy during a 1- to 6-year treatment period reduced the combined end points of mortality and nonfatal reinfarction by approximately 20%.[214–216]

The value of oral anticoagulants is supported by the results of three more recent studies.[120,217,218]

The study by the Sixty-Plus Reinfarction Study Group was limited to patients > 60 years who had been treated with oral anticoagulants for at least 6 months.[217] Although there was a significant reduction in reinfarction and in stroke in patients randomized to receive continuing anticoagulant therapy, the findings were limited by its lack of generalizability as a "stopping trial" in a select age group. The study by Smith and associates[218] had no age restriction and has attracted considerable attention since it showed a 50% reduction in the incidence of the combined outcomes of recurrent infarction, stroke, and mortality. The most recent study, Anticoagulants in the Secondary Prevention of Events in Coronary Thrombosis Trial,[120] also had no age restriction and reported a > 50% reduction in reinfarction and a 40% reduction in stroke.

All three contemporary studies used high-intensity regimens (INR of 2.7 to 4.5 in the first and of 2.8 to 4.8 in the other two) and all reported an increased incidence of bleeding with anticoagulants. Indirect support for the efficacy of oral anticoagulants in patients with coronary artery disease comes from a randomized trial of patients with peripheral arterial disease;[219] compared with an untreated control group, a relatively high-intensity oral anticoagulant regimen (INR, 2.6 to 4.5) produced a significant (50%) reduction in mortality (6.8% per year to 3.3% per year). Recently, the results of the Coumadin Aspirin Reinfarction Study showed that low fixed-dose warfarin therapy (INR < 1.5) plus aspirin therapy, 80 mg, is no more effective than aspirin, 160 mg, in the long-term treatment of AMI.[209]

Prosthetic Heart Valves

To our knowledge, there have been no clinical trials comparing oral anticoagulants with an untreated control group in patients with prosthetic heart valves (for ethical reasons), but a clinical trial has confirmed the clinical impression that anticoagulants are effective in this group of patients. In this study, patients with mechanical prosthetic heart valves who were treated with warfarin for 6 months were randomized to receive warfarin (of uncertain intensity) or one of two aspirin-containing antiplatelet drug regimens.[220] The incidence of thromboembolic complications in the group who continued to take warfarin was significantly less than in either of the two groups who received antiplatelet drugs (relative risk reduction, 60 to 79%). The incidence of bleeding was highest in the warfarin group. The minimum effective intensity of anticoagulant therapy has been evaluated in three studies that compared the efficacy and safety of two levels of intensity of warfarin therapy. The first included only patients with tissue heart valves and showed that the moderate-dose regimen (INR, 2.0 to 2.25) was as effective, but produced less bleeding than the more intense regimen (INR, 2.5 to 4.0).[221] The second study,[222] which included patients with mechanical prosthetic heart valves, compared a very-high-intensity regimen prothrombin ratio (INR, 7.4 to 10.8) with a lower-intensity regimen (INR, 1.9 to 3.6). There was no difference in effectiveness between the two regi-

mens, but the higher-intensity regimen produced significantly more bleeding. The third study,[223] which included patients with mechanical prosthetic valves all of whom received aspirin and dipyridamole, compared the efficacy and safety of a moderate-intensity regimen (INR, 2.0 to 3.0) with a high-intensity regimen (INR, 3.0 to 4.5). There was no difference in efficacy between the two regimens but, as in the previous studies, the high-intensity regimen was associated with a statistically significant increase in bleeding. A more recent randomized trial has shown that the addition of aspirin in a dose of 100 mg/d to warfarin (INR, 3.0 to 4.5) results in a marked improvement in efficacy when compared with warfarin (INR, 3.0 to 4.5) plus placebo.[60] The combined low-dose aspirin and high-intensity warfarin regimen produced a significant and clinically impressive reduction in mortality, in cardiovascular mortality, and in stroke. The improvement in efficacy with low-dose aspirin therapy was associated with an increase in minor bleeding and in a nonsignificant trend for an increase in major bleeding, including cerebral hemorrhage. The details of this study are discussed in the chapter on "Antithrombotic Therapy in Patients With Mechanical and Biological Prosthetic Heart Valves" on page 602S.

In a retrospective study, Cannegieter and associates[122] collected data on 16,081 patients with mechanical heart valves who had been seen at four regional Dutch anticoagulant clinics. All patients were treated with oral anticoagulants with a target range of 3.6 to 4.8. The authors reported that the incidence of embolic events rose sharply when the INR fell below 2.5, while the incidence of bleeding increased sharply when the INR was above 5.0. On the basis of this study, they concluded that the optimal target in patients with mechanical heart valves is 3.0 to 4.0.

Guidelines developed by the European Society of Cardiology[224] related the recommended level of anticoagulant intensity to the severity of thromboembolic risk associated with specific prosthetic heart valves. They recommended an INR of 3.0 to 4.5 for first-generation valves, an INR of 3.0 to 3.5 for second-generation valves in the mitral position, and an INR of 2.5 to 3.0 for second-generation valves in the aortic position.

Atrial Fibrillation

Five trials, all with relatively similar study designs, were performed. Three were carried out in the United States; the SPAF trial,[225] the Boston Area Anticoagulation Trial for Atrial Fibrillation,[226] the Stroke Prevention in Atrial Fibrillation Trial;[227] one trial was carried out in Denmark, the Copenhagen Atrial Fibrillation, Aspirin, Anticoagulation study (AFASAK);[228] and the other was performed in Canada, the Canadian Atrial Fibrillation Anticoagulation study.[229] In two of the trials (AFASAK and SPAF), a third group was randomized to aspirin therapy. To be eligible for these studies, patients were required to be good candidates for anticoagulant therapy. All five of the trials reached consistent conclusions with a pooled 68% risk reduction on intention-to-treat analysis. All trials reached a statistically significant difference, except for the Canadian study that was stopped prematurely after the results of other trials became available.[230] In patients who continued to receive treatment with warfarin, the stroke risk reduction was > 80%.[231] The rates of hemorrhagic complications were low, with little difference between the rate of major or intracranial hemorrhage in the warfarin groups and the control groups. Minor hemorrhage was increased by approximately 3% per year in the warfarin group.[232] Results from the aspirin treatment arms of the two original studies that randomized patients to aspirin therapy were consistent with a small benefit. In the AFASAK study, which used a 75-mg daily dose of aspirin, the 14% reduction in stroke was not statistically significant, while in the SPAF trial, a 325-mg/d dose of aspirin was associated with a 44% risk reduction of stroke.

In the SPAF II[233] trial, warfarin was more effective than aspirin for preventing ischemic stroke. However, this difference was almost entirely offset by a higher rate of intracranial hemorrhage in the warfarin group, particularly patients > 75 years of age who had a rate of intracranial hemorrhage 1.8% per year.

The European Atrial Fibrillation Trial study[234] compared anticoagulant therapy, aspirin, and placebo in patients with atrial fibrillation who had sustained a mild stroke or transient ischemic attack within the last 3 months. There was a 68% reduction in stroke with anticoagulant therapy and a 16% stroke risk reduction in the aspirin group (not significant). Of interest, the reduction in stroke with an aspirin dose of 300 mg/d was comparable to the reduction observed in the AFASAK study that used an aspirin dose of 75 mg/d. None of the patients in the anticoagulant group of the European Atrial Fibrillation Trial suffered an intracranial hemorrhage.

The intensity of anticoagulant therapy was higher in the SPAF trials than in the other primary prevention studies. In addition, most patients who suffered an intracranial hemorrhage during any of the trials had relatively high levels of anticoagulation, estimated INRs > 3.0 at the time of the hemorrhage.[119] Therefore, it is possible that the high rates of cerebral hemorrhage observed in the anticoagulant group in the SPAF II study were related to the high intensity of anticoagulation in the elderly patients who are at particularly high risk for intracranial hemorrhage when INR values are > 3.0.

Since the last communication, the SPAF III study has been published.[210] The results show that warfarin (INR, 2.0 to 3.0) is much more effective than fixed-dose warfarin (3 mg) plus aspirin.

Other Indications

There are other important and well-accepted indications for oral anticoagulant therapy, but the use of oral anticoagulants for these indications has never been evaluated in properly designed clinical trials. Thus, oral anticoagulants have not been compared with an untreated control group or with another antithrombotic regimen in patients with valvular heart disease (with or without atrial fibrillation) or in patients who have suffered at least one episode of systemic embolism. Long-term oral anticoagulant therapy is indicated in patients with valvular heart disease with associated atrial fibrillation and in other

selected patients with mitral stenosis (this is discussed in the chapter on "Antithrombotic Therapy in Valvular Heart Disease" on page 590S). The optimal therapeutic range for this indication is uncertain, but on the basis of current evidence, it would be reasonable to use the moderate-dose regimen of an INR of 2.0 to 3.0. Oral anticoagulants are indicated in patients who have suffered one or more episodes of systemic embolism. The optimal therapeutic range is uncertain. A moderate dose regimen (INR, 2.0 to 3.0) is recommended until further information is available. Anticoagulants are not indicated in patients with nonembolic cerebrovascular disease.[235]

In summary, therefore, there is good evidence that a moderate-dose oral anticoagulant regimen with a targeted therapeutic range of an INR of 2.0 to 3.0 is effective in the prevention of venous thrombosis, in the treatment of venous thrombosis after an initial course of heparin, in the prevention of systemic embolism in patients with AMI, in patients with prosthetic tissue heart valves, and in patients with chronic atrial fibrillation. There is also suggestive evidence that this moderate-dose regimen is effective in preventing systemic embolism in patients with atrial fibrillation who undergo direct current cardioversion. It is generally regarded that a more intense anticoagulant effect is required to provide protection in patients with some mechanical prosthetic heart valves.[69,236,237] This impression is based on a large amount of clinical experience and on the results of a retrospective survey.[237] It has never been confirmed by randomized trial.

Adverse Effects

Bleeding is the main complication of oral anticoagulant therapy. The risk of bleeding is influenced by the intensity of anticoagulant therapy[130,206,221–223,238] (Table 7), by the patient's underlying clinical disorder,[130,239] and by the concomitant use of aspirin, which both impairs platelet function and produces gastric erosions, and when used in very high doses, it impairs synthesis of vitamin K-dependent clotting factors.[54,59]

Four randomized studies have demonstrated that the risk of clinically important bleeding is reduced by lowering the therapeutic range from 3.0 to 4.5 to 2.0 to 3.0.[206,221–223] Although this difference in anticoagulant intensity is associated with a reduction of the mean dose of warfarin of only approximately 1 mg, the effect on bleeding is impressive.

The risk of major bleeding has been reported to be increased by age > 65 years, a history of stroke or GI bleeding, atrial fibrillation, and the presence of serious comorbid conditions such as renal insufficiency or anemia.[130,238] Bleeding that occurs when the INR is < 3.0 is frequently associated with an obvious underlying cause or an occult GI or renal lesion.[238] The hemorrhagic complications of warfarin are discussed in detail in the chapter on "Hemorrhagic Complications of Anticoagulant Therapy," page 511S.

Several studies have reported that the elderly are more prone to bleeding, even after controlling for the intensity of the anticoagulant effect.[121,238] In addition, elderly people show an exaggerated anticoagulant response to warfarin,[240] possibly because the clearance of warfarin declines with age.[241,242] It is prudent, therefore, to initiate warfarin therapy with lower doses in the elderly.

The most important nonhemorrhagic side effect of warfarin is skin necrosis. This uncommon complication is usually observed on the third to eighth day of therapy[243,244] and is caused by extensive thrombosis of the venules and capillaries within the subcutaneous fat. An association has been reported between warfarin-induced skin necrosis and protein C deficiency[245–247] and less commonly, protein S deficiency,[248] but this complication can also occur in nondeficient individuals. The pathogenesis of this striking complication is unknown. A role for protein C deficiency seems probable and is supported by the similarity of the lesions to those seen in neonatal purpura fulminans that complicates homozygous protein C deficiency. The reason for the unusual localization of the lesions remains a mystery. The treatment of patients with warfarin-induced skin necrosis who require life-long anticoagulant therapy is problematic. Warfarin is considered to be contraindicated and long-term heparin therapy is inconvenient and associated with osteoporosis. A reasonable approach in such patients is to restart warfarin therapy at a low dose, *eg*, 2 mg, under the coverage of therapeutic doses of heparin and to increase the warfarin dosage gradually over several weeks. This approach should avoid an abrupt fall in protein C levels before there is a reduction in the levels of factors II, IX, and X and has been shown to be free of recurrence of skin necrosis in a number of case reports.[246,247]

Pregnancy

Oral anticoagulants cross the placenta and can produce a characteristic embryopathy, CNS abnormalities, or fetal bleeding.[21] This complication is discussed in detail in the chapter on "Use of Antithrombotic Agents During Pregnancy," page 524S. Warfarin should not be used in the first trimester of pregnancy and, if possible, it should also be avoided throughout the entire pregnancy. In some cases, however, *eg*, a mechanical heart valve treated with warfarin, where there is a high risk of embolism, and full-dose heparin cannot be used, or where a temporary loss of therapeutic control would be life-threatening, a decision to continue warfarin therapy throughout pregnancy could be justified. Heparin is preferred when anticoagulants are indicated in pregnancy. There is convincing evidence that warfarin does not induce an anticoagulant effect in the breast-fed infant when the drug is administered to a nursing mother.[249,250]

References

1 The Fourth ACCP Consensus Conference on Antithrombotic therapy. Chest 1995; 108(suppl):225S–522S

2 The Medical Research Council's General Practice Research Framework. Thrombosis prevention trial: randomised trial of low-intensity oral anticoagulation with warfarin and low-dose aspirin in the primary prevention is ischaemic heart disease in men at increased risk. Lancet 1998; 351:233–241

3 Whitlon DS, Sadowski JA, Suttie JW. Mechanisms of coumarin action: significance of vitamin K epoxide reductase inhibition. Biochemistry 1978; 17:1371–1377

4 Fasco MJ, Hildebrandt EF, Suttie JW. Evidence that warfarin anticoagulant action involves two distinct reductase activities. J Biol Chem 1982; 257:11210–11212
5 Choonara IA, Malia RG, Haynes BP, et al. The relationship between inhibition of vitamin K 1,2,3-epoxide reductase and reduction of clotting factor activity with warfarin. Br J Clin Pharmacol 1988; 25:1–7
6 Trivedi LS, Rhee M, Galivan JH, et al. Normal and warfarin-resistant rat hepatocyte metabolism of vitamin K 2,3 epoxide: evidence for multiple pathways of hydroxyvitamin K formation. Arch Biochem Biophys 1988; 264:67–73
7 Stenflo J, Fernlund P, Egan W, et al. Vitamin K dependent modifications of glutamic acid residues in prothrombin. Proc Natl Acad Sci USA 1974; 71:2730–2733
8 Nelsestuen GL, Zytkovicz TH, Howard JB. The mode of action of vitamin K. Identification of γ-carboxyglutamic acid as a component of prothrombin. J Biol Chem 1974; 249:6347–6350
9 Nelsestuen GL. Role of gamma-carboxyglutamic acid: an unusual transition required for calcium-dependent binding of prothrombin to phospholipid. J Biol Chem 1976; 251: 5648–5656
10 Prendergast FG, Mann KG. Differentiation of metal ion-induced transitions of prothrombin fragment 1. J Biol Chem 1977; 252:840–850
11 Borowski M, Furie BC, Bauminger S, et al. Prothrombin requires two sequential metal-dependent conformational transitions to bind phospholipid. J Biol Chem 1986; 261: 14969–14975
12 Friedman PA, Rosenberg RD, Hauschka PV, et al. A spectrum of partially carboxylated prothrombins in the plasmas of coumarin treated patients. Biochim Biophys Acta 1977; 494:271–276
13 Malhotra OP, Nesheim ME, Mann KG. The kinetics of activation of normal and gamma carboxy glutamic acid deficient prothrombins. J Biol Chem 1985; 260:279–287
14 Malhotra OP. Dicoumarol induced prothrombins containing 6, 7, and 8 γ-carboxyglutamic acid residues: isolation and characterization. Biochem Cell Biol 1989; 67:411–421
15 Malhotra OP. Dicoumarol induced 9-gamma-carboxyglutamic acid prothrombin: isolation and comparison with 6-, 7-, 8- and 10-gamma carboxyglutamic acid isomers. Biochem Cell Biol 1990; 68:705–715
16 Hauschka PV, Lian JB, Cole DEC, et al. Osteocalcin and matrix Gla protein: vitamin K dependent proteins in bone. Physiol Rev 1989; 69:990–1047
17 Price PA. Role of vitamin K-dependent proteins in bone metabolism. Annu Rev Nutr 1988; 8:565–583
18 Maillard C, Berruyer M, Serre CM, et al. Protein S, a vitamin K-dependent protein is a bone matrix component synthesized and secreted by osteoblasts. Endocrinology 1997; 130:1599–1604
19 Pan LC, Williamson MK, Price PA. Sequence of the precursor to rat bone Y-carboxyglutamic acid protein that accumulated in warfarin-treated osteosarcoma cells. J Biol Chem 1985; 260:13398–13401
20 Pettifor JM, Benson R. Congenital malformations associated with the administration or oral anticoagulants during pregnancy. J Pediatr 1975; 86:459–462
21 Hall JG, Pauli RM, Wilson KM. Maternal and fetal sequelae of anticoagulation during pregnancy. Am J Med 1980; 68:122–140
22 Breckenridge AM. Oral anticoagulant drugs: pharmacokinetic aspects. Semin Hematol 1978; 15:19–26
23 O'Reilly RA. Vitamin K and other oral anticoagulant drugs. Annu Rev Med 1976; 27:245–261
24 Kelly JG, O'Malley K. Clinical pharmacokinetics of oral anticoagulants. Clin Pharmacokinet 1979; 4:1–15
25 O'Reilly RA. Warfarin metabolism and drug-drug interactions. In: Wessler S, Becker CG, Nemerson Y, eds. The new dimensions of warfarin prophylaxis (vol 214). Advances in experimental medicine and biology. New York, NY: Plenum, 1986; 205–212
26 O'Reilly RA, Aggeler PM. Determinants of the response to oral anticoagulant drug in man. Pharmacol Rev 1970; 22:35–96
27 Breckenridge A, Orme M, Wesseling H, et al. Pharmacokinetics and pharmacodynamics of the enantiomers of warfarin in man. Clin Pharmacol Ther 1974; 15:424–430
28 O'Reilly RA. Studies on the optical enantiomorphs of warfarin in man. Clin Pharmacol Ther 1974; 16:348–354
29 O'Reilly RA, Trager WF. Stereoselective interaction of phenylbutazone with $^{13}C/^{12}C$ labelled racemates of warfarin in man [abstract]. Fed Proc 1978; 37:545
30 O'Reilly RA. Lack of effect of fortified wine ingested during fasting and anticoagulant therapy. Arch Intern Med 1981; 141:458–459
31 Toon S, Low LK, Gibaldi M, et al. The warfarin-sulfinpyrazone interaction: stereochemical considerations. Clin Pharmacol Ther 1986; 39:15–24
32 O'Reilly RA. The stereoselective interaction of warfarin and metronidazole in man. N Engl J Med 1976; 295:354–357
33 O'Reilly RA. Stereoselective interaction of trimethoprim-sulfamethoxazole with the separated enantiomorphs of racemic warfarin in man. N Engl J Med 1980; 302:33–35
34 Lewis RJ, Trager WF, Chan KK, et al. Warfarin: stereochemical aspects of its metabolism and the interaction with phenylbutazone. J Clin Invest 1974; 53:1607–1617
35 O'Reilly RA, Trager WF, Rettie AE, et al. Interaction of amiodarone with racemic warfarin and its separated enantiomorphs in humans. Clin Pharmacol Ther 1987; 42:290–294
36 Cropp JS, Bussey HI. A review of enzyme induction of warfarin metabolism with recommendations for patient management. Pharmacotherapy 1997; 17:917–928
37 O'Reilly RA, Pool JG, Aggeler PM. Hereditary resistance to coumarin anticoagulant drugs in man and rat. Ann NY Acad Sci 1968; 151:913–931
38 O'Reilly RA, Aggeler PM, Hoag MS, et al. Hereditary transmission of exceptional resistance to coumarin anticoagulant drugs. N Engl J Med 1983: 308:1229–1230
39 Alving BM, Strickler MP, Knight RD, et al. Hereditary warfarin resistance. Arch Intern Med 1985; 145:499–501
40 O'Reilly R, Rytand D. Resistance to warfarin due to unrecognized vitamin K supplementation. N Engl J Med 1980; 303:160–161
41 Suttie JW, Muhah-Schendel LL, Shah DV, et al. Vitamin K deficiency from dietary vitamin K restriction in humans. Am J Clin Nutr 1988; 47:475–480
42 Bovill EG, Lawson J, Sadowski J, et al. Mechanisms of vitamin K metabolism and vitamin K-dependent hemostasis: implications for warfarin therapy. In: Ezekowitz MD, ed. The heart as a source of systemic embolisation. New York, NY: Dekker, 1992; 31–54
43 Sadowski JA, Booth SL, Mann KG, et al. Structure and mechanism of activation of vitamin K antagonists. In: Poller L, Hirsh J, eds. Oral anticoagulants. London, UK: Arnold, 1996; 9–29
44 Manotti C, Quintavalla R, Pattacini C, et al. Seasonal variation of oral anticoagulant effect. Thromb Haemost 1994; 71:802–803
45 Booth SL, Charnley JM, Saddowski JA, et al. Dietary vitamin K_1 and stability of oral anticoagulation: proposal of a diet with a constant vitamin K_1 content. Thromb Haemost 1997; 77:504–509
46 Richards RK. Influence of fever upon the action of 3,3-methylene bis-(4-hydroxoycoumarin). Science 1943; 97:313–316

47 Owens JC, Neely WB, Owen WR. Effect of sodium dextrothyroxine in patients receiving anticoagulants. N Engl J Med 1962; 266:76–79
48 Bechtold H, Andrassy K, Jahnchen E, et al. Evidence for impaired hepatic vitamin K_1 metabolism in patients treated with N-methyl-thiotetrazole cephalosporins. Thromb Haemost 1984; 51:358–361
49 Weitkamp M, Aber R. Prolonged bleeding times and bleeding diathesis associated with moxalactam administration. JAMA 1983; 249:69–71
50 O'Reilly RA, Sahud MA, Robinson AJ. Studies on the interaction of warfarin and clofibrate in man. Thromb Diath Haemorrh 1972; 27:309–318
51 Hylek EM, Heiman H, Skates SJ, et al. Acetaminophen and other risk factors for excessive warfarin anticoagulation. JAMA 1998; 279:657–662
52 Bell WR. Acetaminophen and warfarin: undesirable synergy. JAMA 1998; 279:702–703
53 Rothschild BM. Commentary: hematological pertubations associated with salicylate. Clin Pharmacol Ther 1979; 26:145–152
54 Dale J, Myhre E, Loew D. Bleeding during acetysalicylic acid and anticoagulant therapy in patients with reduced platelet reactivity after aortic valve replacement. Am Heart J 1980, 99:746–751
55 Schulman S, Henriksson K. Interaction of ibuprofen and warfarin on primary haemostasis. Br J Rheumatol 1989; 38:46–49
56 Casenave J-P, Packham MA, Guccione MA, et al. Effects of penicillin G on platelet aggregation, release and adherence to collagen. Proc Soc Exp Med 1973; 142:159–166
57 Brown CH, Natelson EA, Bradshaw MW, et al. The hemostatic defect produced by carbenicillin. N Engl J Med 1974; 291:265–270
58 Roth CJ, Majerus PW. The mechanism of the effect of aspirin on human platelets: I. Acetylation of a particulate fraction protein. J Clin Invest 1975; 56:624–632
59 Chesebro JH, Fuster V, Elveback LR, et al. Trial of combined warfarin plus dipyridamole or aspirin therapy in prosthetic heart valve replacement: danger of aspirin compared with dipyridamole. Am J Cardiol 1983; 51:1537–1541
60 Turpie AGG, Gent M, Laupacis A, et al. Aspirin and warfarin after heart-valve replacement: a comparison of aspirin with placebo in patients treated with warfarin after heart-valve replacement. N Engl J Med 1993; 329:524–529
61 Weibert RT, Lorentz SM, Townsend RJ, et al. Effect of erythromycin in patients receiving long-term warfarin therapy. Clin Pharm 1989; 8:210–224
62 Lorentz SM, Weibert RT. Potentiation of warfarin anticoagulation by topical testosterone treatment. Clin Pharmacy 1985; 4:332–334
63 Udall JA. Human sources and absorption of vitamin K in relation to anticoagulation. JAMA 1965; 194:127–129
64 Wells PS, Holbrook AM, Crowther NR, et al. The interaction of warfarin with drugs and food: a critical review of the literature. Ann Intern Med 1994; 121:676–683
65 Koch-Weser J, Sellers DM. Drug interactions with oral anticoagulants. N Engl J Med 1971; 285:487–498
66 Sellers DM, Koch-Weser J. Drug interactions with coumarin anticoagulants. N Engl J Med 1971; 285:547–558
67 Quick AJ. The prothrombin time in haemophilia and in obstructive jaundice. J Biol Chem 1935; 109:73–74
68 Zucker S, Cathey MH, Sox PJ, et al. Standardization of laboratory tests for controlling anticoagulant therapy. Am J Clin Pathol 1970; 53:348–354
69 Poller L. Progress in standardization in anticoagulant control. Hematol Rev 1987; 1:225–241
70 Latallo ZS, Thomson JM, Poller L. An evaluation of chromogenic substrates in the control of oral anticoagulant therapy. Br J Haematol 1981; 47:307–318
71 Poller L, Taberner DA. Dosage and control of oral anticoagulants: an international survey. Br J Haematol 1982; 51:479–485
72 Bussey HI, Force RW, Bianco TM, et al. Reliance on prothrombin time ratios causes significant errors in anticoagulation therapy. Arch Intern Med 1992; 152:278–282
73 Tripodi A, Chantarangkul V, Braga M, et al. Results of a multicentre study assessing the status of a recombinant thromboplastin. Thromb Haemost 1994; 72:261–267
74 Wessler S, Gitel SN. Warfarin: from bedside to bench. N Engl J Med 1984; 311:645–652
75 Zivelin A, Rao VM, Rapaport SI. Mechanism of the anticoagulant effect of warfarin as evaluated in rabbits by selective depression of individual procoagulant vitamin-K dependent clotting factors. J Clin Invest 1993; 92:2131–2140
76 Patel P, Weitz J, Brooker LA, et al. Decreased thrombin activity of fibrin clots prepared in cord plasma compared to adult plasma. Pediatr Res 1996; 39:826–830
77 Weitz JI, Hudoba M, Massel D, et al. Clot-bound thrombin is protected from inhibition by heparin-antithrombin III but is susceptible to inactivation by antithrombin III-independent inhibitors. J Clin Invest 1990; 86:385–391
78 Furie B, Diuguid CF, Jacobs M, et al. Randomized prospective trial comparing the native prothrombin antigen with the prothrombin time for monitoring anticoagulant therapy. Blood 1990; 75:344–349
79 Harrison L, Johnston M, Massicotte MP, et al. Comparison of 5-mg and 10-mg loading doses in initiation of warfarin therapy. Ann Intern Med 1997; 126:133–136
80 Kirkwood TBL. Calibration of reference thromboplastins and standardisation of the prothrombin time ratio. Thromb Haemost 1983; 49:238–244
81 Hirsh J. Is the dose of warfarin prescribed by American physicians unnecessarily high? Arch Intern Med 1987; 147: 769–771
82 Poller L. A simple nomogram for the derivation of international normalised ratios for the standardisation of prothrombin time. Thromb Haemost 1988; 60:18–20
83 College of American Pathologists Coagulation Survey Set 1997 CG2-C. Northfield, IL: College of American Pathologists, 1997
84 Poller L. The effect of the use of different tissue extracts on one-stage prothrombin times. Acta Haematol 1964; 32:292–298
85 Zucker S, Brosious E, Cooper JR. One-stage prothrombin time survey. Am J Clin Pathol 1970; 53:340–347
86 McKernan A, Thomson JM, Poller L. The reliability of international normalized ratio during short-term oral anticoagulant treatment. Clin Lab Haemost 1988; 10:63–71
87 WHO Expert Committee on Biological Standardization. 33rd report. Technical Report Series No. 687, Geneva 81: WHO, 1983
88 Johnston M, Harrison L, Moffat K, et al. Reliability of the international normalized ratio for monitoring the induction phase of warfarin: comparison with the prothrombin time ratio. J Lab Clin Med 1996; 128:214–217
89 Kovacs MJ, Wong A, MacKinnon K, et al. Assessment of the validity of the INR system for patients with liver impairment. Thromb Haemost 1994; 71:727–730
90 Taberner DA, Poller L, Thomson JM, et al. Effect of international sensitivity index (ISI) of thromboplastins on precision of international normalized ratios (INR). J Clin Pathol 1989; 42:92–96
91 Poller L, Hirsh J. Laboratory monitoring of anticoagulants. In: Poller L, Hirsh J, eds. Oral anticoagulants. London, UK: Arnold, 1996; 49–64
92 Brien WF, Crawford L, Wood DE. Discrepant results in

INR testing. Thromb Haemost 1994; 72:986–987
93 van den Besselaar AMHP, Evatt BL, Brogan DR, et al. Proficiency testing and standardization of prothrombin time: effect of thromboplastin, instrumentation, and plasma. Am J Clin Pathol 1984; 82:688–699
94 Poller L, Triplett DA, Hirsh J, et al. The value of plasma calibrants in correcting coagulometer effects on international normalized ratios—an international multicenter study. Am J Clin Pathol 1995; 103:358–365
95 Poggio M, van den Besselaar AMHP, van der Velde EA, et al. The effect of some instruments for prothrombin time testing on the international sensitivity index (ISI) of two rabbit tissue thromboplastin reagents. Thromb Haemost 1989; 62:868–874
96 D'Angelo A, Seveso MP, D'Angelo SV, et al. Comparison of two automated coagulometers and the manual tilt tube method for determination of prothrombin time. Am J Clin Pathol 1989; 92:321–328
97 Poller L, Thomson JM, Taberner DA. Effect of automation on the prothrombin time test in NEQAS surveys. J Clin Pathol 1989; 42:97–100
98 Ray MJ, Smith IR. The dependence of the international sensitivity index on the coagulometer used to perform the prothrombin time. Thromb Haemost 1990; 63:424–429
99 van Rijn JLML, Schmidt NA, Rutten W. Correlation of instrument and reagent based differences in determination of the international normalised ratio (INR) for monitoring anticoagulant therapy. Clin Chem 1989; 355:840
100 Thomson JM, Taberner DA, Poller L. Automation and prothrombin time: a United Kingdom field study of two widely used coagulometers. J Clin Pathol 1990; 43:679–684
101 Poller L, Thomson JM, Taberner DA, et al. The correction of coagulometer effects on international normalized ratio: a multicentre evaluation. Br J Haematol 1994; 86:112–117
102 Poller L, Triplett DA, Hirsh J, et al. A comparison of lyophilized artificially depleted plasma and lyophilized plasma from warfarin treated in correcting for coagulometer effects on international normalized ratios. Am J Clin Pathol 1995; 103:366–371
103 Fairweather RB, Ansell J, van den Besselaar AMHP, et al. Laboratory monitoring of oral anticoagulant therapy. Arch Pathol Lab Med 1998; 122:768–781
104 Ng VL, Levin J, Corash L, et al. Failure of the international normalized ratio to generate consistent results within a local medical community. Am J Clin Pathol 1993; 99:689–694
105 Poller L. Laboratory control of oral anticoagulants. BMJ 1987; 294:1184
106 Kazama M, Suzuki S, Abe T, et al. Evaluation of international normalized ratios by a controlled field survey with four different thromboplastin reagents. Thromb Haemost 1990; 64:535–541
107 van den Besselaar AMHP, Lewis SM, Mannucci PM. Status of present and candidate international reference preparations (IRP) of thromboplastin for the prothrombin time: a report of the Subcommittee for Control of Anticoagulation. Thromb Haemost 1993; 69:85
108 Duncan EM, Casey CR, Duncan BM, et al. Effect of concentration of trisodium citrate anticoagulant on calculation of the international normalized ratio and the international sensitivity index of thromboplastin. Thromb Haemost 1994; 72:84–88
109 Tripodi A, Clerici M, Negri B, et al. Influence of citrate concentration on the international sensitivity index of five thromboplastins [abstract 1287]. Thromb Haemost 1995; 73:1237
110 Adcock DM, Kressen DC, Marlar RA. Effect of 3.2% vs 3.8% sodium citrate on routine coagulation testing. Am J Clin Pathol 1997; 107:105–110
111 Rosove MH, Brever PM. Antiphospholipid thrombosis; clinical course after the first thrombotic event in 70 patients. Ann Intern Med 1992; 117:303–308
112 Khamashta ML, Cuadrado MJ, Mujic F, et al. The management of thrombosis in the antiphospholipid-antibody syndrome. N Engl J Med 1995; 332:993–997
113 Moll S, Ortel TL. Monitoring warfarin therapy in patients with lupus anticoagulants. Ann Intern Med 1997; 127:177–185
114 Della Valle P, Crippa L, Safa O, et al. Potential failure of the international normalized ratio (INR) system in the monitoring of oral anticoagulation in patients with lupus anticoagulants. Ann Med Interne (Paris) 1996;147(suppl):10–14
115 Rapaport SI, Le DT. Thrombosis in the antiphospholipid antibody syndrome [letter]. N Engl J Med 1995; 333:665
116 Le DT, Weibert RT, Sevilla BK, et al. The international normalized ratio (INR) for monitoring warfarin therapy: reliability and relation to other monitoring methods. Ann Intern Med 1994; 120:552–558
117 Lind SE, Callas PW, Golden EA, et al. Plasma levels of factors II, VII, and X and their relationship to the international normalized ratio during chronic warfarin therapy. Blood Coagul Fibrinolysis 1997; 8:48–53
118 Kornbert A, Francis DW, Pellegrini VD Jr, et al. Comparison of native prothrombin antigen with the prothrombin time for monitoring oral anticoagulant prophylaxis. Circulation 1993; 88:454–460
119 Alberts GW. Atrial fibrillation and stroke: three new studies remaining questions. Arch Intern Med 1994; 154:1443–1448
120 ASPECT Research Group. Effect of long-term oral anticoagulant treatment on mortality and cardiovascular morbidity after myocardial infarction. Lancet 1994; 343:499–503
121 Hylek EM, Singer DE. Risk factors for intracranial hemorrhage in outpatients taking warfarin. Ann Intern Med 1994; 120:897–902
122 Cannegieter SC, Rosendaal FR, Wintzen AR, et al. The optimal intensity of oral anticoagulant therapy in patients with mechanical heart valve prostheses: the Leiden artificial valve and anticoagulation study. N Engl J Med 1995; 333:11–17
123 A randomized trial of anticoagulants versus aspirin after cerebral ischemia of presumed arterial origin: the Stroke Prevention in Reversible Ischemia Trial (SPIRIT) study group. Ann Neurol 1997; 42:857–865
124 Ansell JE, Hughes R. Evolving models of warfarin management: anticoagulation clinics, patient self-monitoring, and patient self-management. Am Heart J 1996; 132:1095–1100
125 Ansell JE, Buttaro ML, Boltis-Thomas O, et al. Consensus guidelines for coordinated outpatient oral anticoagulation therapy management. Ann Pharmacother 1997; 31:604–615
126 Garabedian-Ruffalo SM, Gray DR, Sax MJ, et al. Retrospective evaluation of a pharmacist-managed warfarin anticoagulation clinic. Am J Hosp Pharm 1985; 42:304–308
127 Cortelazzo S, Finazzi G, Viero P, et al. Thrombotic and hemorrhagic complications in patients with mechanical heart valve prostheses attending an anticoagulation clinic. Thromb Haemost 1993; 69:316–320
128 Wilt VM, Gums JG, Ahmed OI, et al. Pharmacy operated anticoagulation service: improved outcomes in patients on warfarin. Pharmacotherapy 1995; 15:732–779
129 Chiquette E, Amato MG, Bussey HI. Comparison of an anticoagulation clinic and usual medical care: anticoagulation control, patient outcomes, and health care costs. Arch Intern Med 1998; 158:1641–1647
130 Landefeld CS, Goldman L. Major bleeding in outpatients treated with warfarin: incidence and prediction by factors known at the start of outpatient therapy. Am J Med 1989; 87:144–152
131 Gitter MJ, Jaeger TM, Petterson TM, et al. Bleeding and

thromboembolism during anticoagulant therapy: a population-based study in Rochester, Minnesota. Mayo Clin Proc 1995; 70:725–733
132 Forfar JC. Prediction of hemorrhage during long-term oral coumarin anticoagulation by excessive prothrombin ratio. Am Heart J 1982; 103:445–446
133 Errichetti AM, Holden A, Ansell J. Management of oral anticoagulant therapy: experience with an anticoagulation clinic. Arch Intern Med 1984; 144:1966–1968
134 Conte RR, Kehoe WA, Nielson N, et al. Nine-year experience with a pharmacist-managed anticoagulation clinic. Am J Hosp Pharm 1986; 43:2460–2464
135 Petty GW, Lennihan L, Mohr JP, et al. Complications of long-term anticoagulation. Ann Neurol 1988; 23:570–574
136 Charney R, Leddomado E, Rose DN, et al. Anticoagulation clinics and the monitoring of anticoagulant therapy. Int J Cardiol 1988; 18:197–206
137 Bussey HI, Rospond RM, Quandt CM, et al. The safety and effectiveness of long-term warfarin therapy in an anticoagulation clinic. Pharmacotherapy 1989; 9:214–219
138 Seabrook GR, Karp D, Schmitt DD, et al. An outpatient anticoagulation protocol managed by a vascular nurse-clinician. Am J Surg 1990; 160:501–504
139 Fihn SD, McDonell M, Martin D, et al. Risk factors for complications of chronic anticoagulation: a multicenter study. Ann Intern Med 1993; 118:511–520
140 van der Meer FJ, Rosendaal FR, Vandenbrouke JP, et al. Bleeding complications in oral anticoagulant therapy: an analysis of risk factors. Arch Intern Med 1993; 153:1557–1562
141 Palareti G, Leali N, Coccheri S, et al. Bleeding complications of oral anticoagulant treatment: an inception-cohort, prospective collaborative study (ISCOAT). Lancet 1996; 348:423–428
142 Palareti G, Manotti C, D'Angelo A, et al. Thrombotic events during anticoagulant treatment: results of the inception-cohort, prospective, collaborative ISCOAT study. Thromb Haemost 1997; 78:1438–1443
143 Gray DR, Garabedian-Ruffalo SM, Chretien SD. Cost justification of a clinical pharmacist-managed anticoagulation clinic. Drug Intel Clin Pharm 1985; 19:575–580
144 Leaning KE, Ansell JE. Advances in the monitoring of oral anticoagulation: point-of-care testing, patient self-monitoring, and patient self-management. J Thrombo Thrombolys 1996; 3:377–383
145 Lucas FV, Duncan A, Jay R, et al. A novel whole blood capillary technique for measuring prothrombin time. Am J Clin Pathol 1987; 88:442–446
146 Yano Y, Kambayashi J, Murata K, et al. Bedside monitoring of warfarin therapy by a whole blood capillary coagulation monitor. Thromb Res 1992; 66:583–590
147 Weibert RT, Adler DS. Evaluation of a capillary whole blood prothrombin time measurement system. Clin Pharm 1989; 8:864–867
148 Jennings I, Luddington RJ, Baglin T. Evaluation of the Cibra Corning Biotrack 512 coagulation monitor for the control of oral anticoagulation. J Clin Pathol 1991; 44:950–953
149 McCurdy SA, White RH. Accuracy and precision of a portable anticoagulation monitor in a clinical setting. Arch Intern Med 1992; 152:589–592
150 Tripodi A, Arbini AA, Chantarangkul V, et al. Are capillary whole blood coagulation monitors suitable for the control of oral anticoagulant treatment by the international normalized ratio? Thromb Haemost 1993; 70:921–924
151 Oberhardt BJ, Dermott SC, Taylor M, et al. Dry reagent technology for rapid, convenient measurements of blood coagulation and fibrinolysis. Clin Chem 1991; 37:520–526
152 Rose VL, Dermott SC, Murray BF, et al. Decentralized testing for prothrombin time and activated partial thromboplastin time using a dry chemistry portable analyzer. Arch Pathol Lab Med 1993; 117:611–617
153 Fabbrini N, Messmore H, Balbale S, et al. Pilot study to determine use of a TAS analyzer in an anticoagulation clinic setting. Blood 1995; 86(suppl 1):869a
154 Tripodi A, Chantarangkul V, Clerici M, et al. Determination of the international sensitivity index of a new near-patient testing device to monitor oral anticoagulant therapy. Thromb Haemost 1997; 78:855–858
155 Kaatz AA, White RH, Hill J, et al. Accuracy of laboratory and portable monitor international normalized ratio determinations. Arch Intern Med 1995; 155:1861–1867
156 Ansell J, Becker D, Andrew M, et al. Accurate and precise prothrombin time measurement in a multicenter anticoagulation trial employing patient self-testing. Blood 1995; 86(suppl 1):864a
157 Andrew M, Marzinotto V, Adams M, et al. Monitoring of oral anticoagulant therapy in pediatric patients using a new microsample PT device. Blood 1995; 86(suppl 1):863a
158 Anderson D, Harrison L, Hirsh L. Evaluation of a portable prothrombin time monitor for home use by patients who require long-term oral anticoagulant therapy. Arch Intern Med 1993; 153:1441–1447
159 White RN, McKittrick T, Hutchinson R, et al. Temporary discontinuation of warfarin therapy: changes in the international normalized ratio. Ann Intern Med 1995; 122:40–42
160 Beyth RJ, Landefeld CS. Prevention of major bleeding in older patients treated with warfarin: results of a randomized trial [abstract]. J Gen Intern Med 1997; 12:66
161 Erdman S, Vidne B, Levy MJ. A self-control method for long-term anticoagulation therapy. J Cardiovasc Surg 1974; 15:454–457
162 Ansell J, Holden A, Knapic N. Patient self-management of oral anticoagulation guided by capillary (fingerstick) whole blood prothrombin times. Arch Intern Med 1989; 149:2509–2511
163 Ansell J, Patel N, Ostrovsky D, et al. Long-term patient self-management of oral anticoagulation. Arch Intern Med 1995; 155:2185–2189
164 Bernardo A. Experience with patient self-management of oral anticoagulation. J Thromb Thrombolys 1996; 2:321–325
165 Horstkotte D, Piper C, Wiemer M, et al. Improvement of prognosis by home prothrombin estimation in patients with life-long anticoagulant therapy [abstract]. Eur Heart J 1996; 17(suppl):230
166 Wilson R, James AH. Computer assisted management of warfarin treatment. BMJ 1984; 289:422–424
167 Wyld PJ, West D, Wilson TH. Computer dosing in anticoagulant clinics—the way forward? Clin Lab Haematol 1988; 10:235–236
168 Ryan PJ, Gilbert M, Rose PE. Computer control of anticoagulant dose for therapeutic management. BMJ 1989; 299: 1207–1209
169 Weston Smith SG, Savidge GF. Computer control of anticoagulant dose. BMJ 1989; 299:1529
170 Poller L, Wright D, Rowlands M. Prospective comparative study of computer programs used for management of warfarin. J Clin Pathol 1993; 46:299–303
171 Ageno W, Turpie AGG. A randomized comparison of a computer-based dosing program with a manual system to monitor oral anticoagulant therapy. J Thrombo Thrombolys 1998; 5(suppl 1):S69
172 Poller L, Shiach CR, MacCallum P, et al. Computerised anticoagulant dosage—a multicentre randomised study. Lancet 1998 (in press)
173 Whitling AM, Bussey HI, Lyons RM. Comparing different routes and doses of vitamin K1 for reversing excessive anticoagulation. Arch Intern Med 1998 (in press)

174 Cosgriff SW. The effectiveness of an oral vitamin K_1 in controlling excessive hypoprothrombinemia during anticoagulant therapy. Ann Intern Med 1956; 45:14–22
175 Shirger A, Spittel JA, Ragen PA. Small doses of vitamin K_1 for correction of reduced prothrombin activity. Proc Mayo Clin 1959; 34:453–458
176 van der Meer J, Hemker HC, Loeliger EA. Pharmacological aspects of vitamin K_1: a clinical and experimental study in man. Thrombos Diathes Haemorrh Suppl 1968; 29:1–96
177 Weibert RT, Le DT, Kayser SR, et al. Correction of excessive anticoagulation with low-dose oral vitamin K_1. Ann Intern Med 1997; 125:959–962
178 Park BK, Scott AK, Wilson AC, et al. Plasma disposition of vitamin K_1 in relation to anticoagulant poisoning. Br J Clin Pharmacol 1984; 18:655–662
179 Pengo V, Banzato A, Garelli E, et al. Reversal of excessive effect of regular anticoagulation: low oral dose of phytonadion (vitamin K_1) compared with warfarin discontinuation. Blood Coagul Fibrinolysis 1993; 4:739–741
180 Crowther MA, Donovan D, Harrison L, et al. Low-dose oral vitamin K reliably reverses over-anticoagulation due to warfarin. Thromb Haemost 1998; 79:1116–1118
181 Kearon C, Hirsh J. Management of anticoagulation before and after elective surgery. N Engl J Med 1997; 336:1506–1511
182 Sindet-Pedersen S, Ramstrom G, Bernvil S, et al. Hemostatic effect of tranexamic mouthwash in anticoagulant-treated patients undergoing oral surgery. N Engl J Med 1989; 324:840–843
183 Soute JC, Oliver A, ZuaZu-Jausoro I, et al. Oral surgery in anticoagulated patients without reducing the dose of oral anticoagulant: a prospective randomized study. J Oral Maxillofac Surg 1996; 54:27–32
184 Hylek EM, Skates SJ, Sheehan MA, et al. An analysis of the lowest effective intensity of prophylactic anticoagulation for patients with nonrheumatic atrial fibrillation. N Engl J Med 1996; 335:540–546
185 Sevitt S, Gallagher NG. Prevention of venous thrombosis and pulmonary embolism in injured patients. Lancet 1959; 2:981–989
186 Francis CW, Marder VJ, Evarts CM, et al. Two-step warfarin therapy: prevention of postoperative venous thrombosis without excessive bleeding. JAMA 1983; 249:374–378
187 Powers PJ, Gent M, Jay RM, et al. A randomized trial of less intense postoperative warfarin or aspirin therapy in the prevention of venous thromboembolism after surgery for fractured hip. Arch Intern Med 1989; 149:771–774
188 Taberner DA, Poller L, Burslem RW, et al. Oral anticoagulants controlled by the British comparative thromboplastin versus low-dose heparin in prophylaxis of deep vein thrombosis. BMJ 1978; 1:272–274
189 Poller L, McKernan A, Thomson JM, et al. Fixed minidose warfarin: a new approach to prophylaxis against venous thrombosis after major surgery. BMJ 1987; 295:1309–1312
190 Hull R, Raskob G, Pineo G, et al. A comparison of subcutaneous low-molecular-weight heparin with warfarin sodium for prophylaxis against deep-vein thrombosis after hip or knee implantation. N Engl J Med 1993; 19:1370–1375
191 RD Heparin Arthroplasty Group. RD Heparin compared with warfarin for prevention of venous thromboembolic disease following total hip or knee arthroplasty. J Bone Joint Surg 1994; 76A:1174–1185
192 Bern MM, Lokich JJ, Wallach SR, et al. Very low doses of warfarin can prevent thrombosis in central venous catheters. Ann Intern Med 1990; 112:423–428
193 MacCallum PK, Thomson JM, Poller L. Effects of fixed minidose warfarin on coagulation and fibrinolysis following major gynecological surgery. Thromb Haemost 1990; 64:511–515
194 Poller L, MacCallum PK, Thompson JM, et al. Reduction of factor VII coagulant activity (VIIC), a risk factor for ischaemic heart disease, by fixed dose warfarin: a double blind crossover study. Br Heart J 1990; 63:231–233
195 Dale C, Gallus AS, Wycherley A, et al. Prevention of venous thrombosis with minidose warfarin after joint replacement. BMJ 1991; 303:224
196 Fordyce MJF, Baker AS, Staddon GE. Efficacy of fixed minidose warfarin prophylaxis in total hip replacement. BMJ 1991; 303:219–220
197 Poller L, Thomson JM, McCallum PK, et al. Minidose warfarin and failure to prevent deep vein thrombosis after joint replacement surgery despite inhibiting the postoperative rise in plasminogen activator activity. Clin Appl Throm Hemostas 1995; 1:267–273
198 Levine M, Hirsh J, Gent M, et al. Double-blind randomised trial of a very-low-dose warfarin for prevention of thromboembolism in stage IV breast cancer. Lancet 1994; 343:886–889
199 Hirsh J. The optimal duration of anticoagulant therapy for venous thrombosis. N Engl J Med 1995; 332:1710–1711
200 Hull R, Delmore T, Genton E, et al. Warfarin sodium versus low-dose heparin in the long-term treatment of venous thrombosis. N Engl J Med 1979; 302:855–858
201 Hull R, Delmore T, Carter C, et al. Adjusted subcutaneous heparin versus warfarin sodium in the long-term treatment of venous thrombosis. N Engl J Med 1982; 306:189–194
202 Lagerstedt CI, Fagher BO, Albrechtsson U, et al. Need for long-term anticoagulant treatment in symptomatic calf-vein thrombosis. Lancet 1985; 2:515–518
203 Schulman S, Granqvist S, Holmstrom M, et al. The duration of oral anticoagulant therapy after a second episode of venous thromboembolism. N Engl J Med 1997; 336:393–398
204 Schulman S, Rhedin A, Lindmarker P, et al. A comparison of 6 weeks with 6 months of oral anticoagulant therapy after a first episode of venous thromboembolism. N Engl J Med 1995; 332:1661–1665
205 Kearon C, for the LAFIT Investigators. Two years of warfarin versus placebo following 3 months of anticoagulation for a first episode of idiopathic venous thromboembolism (VTE). Thromb Haemostat 1997; 78(suppl):767
206 Hull R, Hirsh J, Jay R, et al. Different intensities of oral anticoagulant therapy in the treatment of proximal-vein thrombosis. N Engl J Med 1982; 307:1676–1681
207 Hull RD, Raskob GE, Rosenbloom D, et al. Heparin for 5 days as compared with 10 days in the initial treatment of proximal venous thrombosis. N Engl J Med 1990; 322:1260–1264
208 Gallus AS, Jackaman J, Tillett J, et al. Safety and efficacy of warfarin started early after submassive venous thrombosis or pulmonary embolism. Lancet 1986; 2:1293–1296
209 CARS Study Investigators. Randomised double-blind trial of fixed low-dose warfarin with aspirin after myocardial infarction (Coumadin Aspirin Reinfarction Study). Lancet 1997; 350:389–396
210 Stroke Prevention in Atrial Fibrillation Investigators (SPAF III). Adjusted dose warfarin versus low-intensity, fixed-dose warfarin plus aspirin for high-risk patients with atrial fibrillation: stroke prevention in atrial fibrillation III randomised clinical trial. Lancet 1996; 348:633–638
211 Veterans Administration Cooperative Study. Anticoagulants in acute myocardial infarction: results of a cooperative clinical trial. JAMA 1973; 225:724–729
212 Drapkin A, Merskey C. Anticoagulant therapy after acute myocardial infarction. JAMA 1972; 222:541–548
213 Medical Research Council Group. Assessment of short-term anticoagulant administration after cardiac infarction: report of the Working Party on Anticoagulant Therapy in Coronary Thrombosis. BMJ 1969; 1:335–342
214 Cairns JA, Hirsh J, Lewis HD Jr, et al. Antithrombotic agents

in coronary artery disease. Chest 1992; 102:456S–481S
215 Goldberg RJ, Gore JM, Dalen JE, et al. Long-term anticoagulant therapy after acute myocardial infarction. Am Heart J 1985; 109:616–622
216 Leizorovicz A, Boissel JP. Oral anticoagulant in patients surviving myocardial infarction. Eur J Clin Pharmacol 1983; 24:333–336
217 Sixty-Plus Reinfarction Study Group. A double-blind trial to assess long-term oral anticoagulants therapy in elderly patients after myocardial infarction. Lancet 1980; 2:989–994
218 Smith P, Arnesen H, Holme I. The effect of warfarin on mortality and reinfarction after myocardial infarction. N Engl J Med 1990; 323:147–151
219 Kretschmer G, Wenzl E, Schemper M, et al. Influence of postoperative anticoagulant treatment of patient survival after femoropopliteal vein bypass surgery. Lancet 1988; 1:797–798
220 Mok CK, Boey J, Wang R, et al. Warfarin versus dipyridamole-aspirin and pentoxifylline-aspirin for the prevention of prosthetic heart valve thromboembolism: a prospective clinical trial. Circulation 1985; 72:1059–1063
221 Turpie AGG, Gunstensen J, Hirsh J, et al. Randomized comparison of two intensities of oral anticoagulant therapy after tissue heart valve replacement. Lancet 1988; 1:1242–1245
222 Saour JN, Sieck JO, Mamo LAR, et al. Trial of different intensities of anticoagulation in patients with prosthetic heart valves. N Engl J Med 1990; 322:428–432
223 Altman R, Rouvier J, Gurfinkel E, et al. Comparison of two levels of anticoagulant therapy in patients with substitute heart valves. J Thorac Cardiovasc Surg 1991; 101:427–431
224 Gohlke-Barwolf C, Acar J, Oakley C, et al. Guidelines for prevention of thromboembolic events in valvular heart disease. Eur Heart J 1995; 16:1320–1330
225 The Stroke Prevention in Atrial Fibrillation Investigators. The stroke prevention in atrial fibrillation study: final results. Circulation 1991; 84:527–539
226 The Boston Area Anticoagulation Trial for Atrial Fibrillation Investigators. The effect of low-dose warfarin on the risk of stroke in patients with nonrheumatic atrial fibrillation. N Engl J Med 1990; 323:1505–1511
227 Ezekowitz MD, Bridgers SL, James KE, et al. Interim analysis of VA Co-operative Study, Stroke Prevention in Non-Rheumatic Atrial Fibrillation (SPINAF). N Engl J Med 1992; 327:1406–1412
228 Petersen P, Boysan G, Godtfredsen J, et al. Placebo-controlled, randomised trial of warfarin and aspirin for prevention of thromboembolic complications in chronic atrial fibrillation: the Copenhagen AFASAK Study. Lancet 1989; 1:175–179
229 Connolly SJ, Laupacis A, Gent M, et al. Canadian Atrial Fibrillation Anticoagulation (CAFA) Study. J Am Coll Cardiol 1991; 18:349–355
230 Atrial Fibrillation Investigators. Risk factors for stroke and efficacy of antithrombotic therapy in atrial fibrillation: analysis of pooled data from five randomized controlled trials. Arch Intern Med 1994; 154:1449–1457
231 Albers GW, Sherman DG, Gress DR, et al. Stroke prevention in nonvalvular atrial fibrillation: a review of prospective randomized trials. Ann Neurol 1991; 30:511–518
232 Atwood J, Albers G. Anticoagulation and atrial fibrillation. Herz 1993; 18:27–38
233 Stroke Prevention in Atrial Fibrillation Investigators. Warfarin versus aspirin for prevention of thromboembolism in atrial fibrillation: Stroke Prevention in Atrial Fibrillation II Study. Lancet 1994; 343:687–691
234 European Atrial Fibrillation Trial Study Group. Secondary prevention in non-rheumatic atrial fibrillation after transient ischaemic attack or minor stroke. Lancet 1993; 342:1255–1262
235 Sherman DG, Dyken ML, Gent M, et al. Antithrombotic therapy for cerebrovascular disorders: an update. Chest 1995; 108:444S–456S
236 Loeliger EA, Poller L, Samama M, et al. Questions and answers on prothrombin time standardisation in oral anticoagulant control. Thromb Haemost 1984; 54:514–518
237 Fuster V, Pumphrey CW, McGoon MD, et al. Systemic thromboembolism in mitral and aortic Starr-Edwards prosthesis: a long term follow-up (10–19 years). Circulation 1982; 66(suppl 1):157–161
238 Landefeld CS, Rosenblatt MW. Bleeding in outpatients treated with warfarin: relation to the prothrombin time and important remediable lesions. Am J Med 1989; 87:153–159
239 Levine MN, Raskob G, Landefeld S, et al. Hemorrhagic complications of anticoagulant treatment. Chest 1995; 108: 276s–290s
240 Gurwitz JH, Avorn J, Ross-Degnan D, et al. Aging and the anticoagulant response to warfarin therapy. Ann Intern Med 1992; 116:901–904
241 Mungall D, White R. Aging and warfarin therapy. Ann Intern Med 1992; 117:878–879
242 Bowles SK. Stereoselective disposition of warfarin in young and elderly subjects. Clin Pharm Ther 1994; 55:172
243 Verhagen H. Local hemorrhage and necrosis of the skin and underlying tissues at starting therapy with dicumarol or dicumacyl. Acta Med Scand 1954; 148:455–467
244 Weinberg AC, Lieskovsky G, McGehee WG, et al. Warfarin necrosis of the skin and subcutaneous tissue of the male genitalia. J Urol 1983; 130:352–354
245 Broekmans AW, Bertina RM, Loeliger EA, et al. Protein C and the development of skin necrosis during anticoagulant therapy. Thromb Haemost 1983; 49:244–251
246 Zauber NP, Stark MW. Successful warfarin anticoagulant despite protein C deficiency and a history of warfarin necrosis. Ann Intern Med 1986; 104:659–660
247 Samama M, Horellou MH, Soria J, et al. Successful progressive anticoagulation in a severe protein C deficiency and previous skin necrosis at the initiation of oral anticoagulation treatment. Thromb Haemost 1984; 51:332–333
248 Grimaudo V, Gueissaz F, Hauert J, et al. Necrosis of skin induced by coumarin in a patient deficient in protein S. BMJ 1989; 298:233–234
249 McKenna R, Cale ER, Vasan U. Is warfarin sodium contraindicated in the lactating mother? J Pediatr 1983; 103:325–327
250 Lao TT, DeSwiet M, Letsky E, et al. Prophylaxis of thromboembolism in pregnancy: an alternative. Br J Obstet Gynaecol 1985; 92:202–206

Endotoxin and thrombin elevate rodent endothelial cell protein C receptor mRNA levels and increase receptor shedding in vivo

Jian-Ming Gu, Yasuhiro Katsuura, Gary L. Ferrell, Paula Grammas, and Charles T. Esmon

The endothelial cell protein C receptor (EPCR) facilitates protein C activation by the thrombin-thrombomodulin complex. Protein C activation has been shown to be critical to the host defense against septic shock. In cell culture, tumor necrosis factor-α (TNF-α) down-regulates EPCR expression, raising the possibility that EPCR might be down-regulated in septic shock. We examined EPCR mRNA and soluble EPCR levels in mice and rats challenged with lethal dose 95 levels of endotoxin. Toxic doses of TNF-α failed to alter EPCR mRNA levels in mice. Rather than EPCR mRNA levels falling in response to endotoxin, as predicted from cell-culture experiments, they rose approximately 3-fold 6 hours after exposure to endotoxin before returning toward baseline levels at 24 hours after exposure. Soluble EPCR levels rose approximately 4-fold. Infusion of hirudin, a specific thrombin inhibitor, before endotoxin exposure almost completely blocked the increase in EPCR mRNA and soluble EPCR. Consistent with the idea that the responses were mediated by thrombin, thrombin infusion (5 U/kg of body weight for 3 hours) resulted in an approximately 2-fold increase in EPCR mRNA and soluble EPCR. Incubation of rat endothelial cells with thrombin or murine protease-activated receptor 1 agonist peptide resulted in a 2-fold increase in EPCR mRNA. These results indicate that thrombin plays a major role in up-regulating EPCR mRNA and shedding in vivo. (Blood. 2000;95:1687-1693)

Introduction

The protein C anticoagulant pathway provides a critical, on-demand mechanism for regulation of blood coagulation (reviewed by Esmon[1]). The pathway is initiated when thrombin binds to thrombomodulin on the surface of the endothelium, and this complex catalyzes protein C activation. Activated protein C (APC) functions as an anticoagulant by proteolytic inactivation of the coagulation cofactors, factor Va and factor VIIIa. Patients with protein C deficiency usually have life-threatening thrombotic complications in infancy[2] that can be corrected by administration of protein C.[3] In addition to modulating the coagulation response, the protein C anticoagulant pathway also appears to modulate the inflammatory response. In vivo, APC administration prevented the lethal effects of *Escherichia coli* infusion in a baboon model of gram-negative sepsis,[4] and preliminary clinical results suggest that protein C is effective in treating certain forms of septic shock.[5-7] In vitro, APC has been reported to inhibit endotoxin-induced tumor necrosis factor-α (TNF-α) elaboration by monocytes[8] and to inhibit leukocyte adhesion to selectins.[9] The exact mechanism by which the protein C pathway modulates inflammatory responses remains unknown.

In an effort to gain further insights into the mechanisms by which APC might modulate inflammation, we sought to identify candidate protein C/APC receptors. In pursuit of this goal, we[10] and others[11] identified high-affinity binding sites for protein C and APC on vascular endothelium. The responsible glycoprotein, named the endothelial cell protein C receptor (EPCR), was identified by expression cloning and was suggested to be a member of the CD1-major histocompatibility complex class I family of molecules on the basis of sequence homology.[10] Protein C binding to EPCR augments protein C activation by the thrombin-thrombomodulin complex on the cell surface.[12] Binding of APC to soluble forms of EPCR blocks the APC anticoagulant activity and the ability of APC to inactivate factor Va without altering sensitivity to inhibition by protein C inhibitor and α_1-antitrypsin.[13] Presumably, this inhibition of APC anticoagulant activity reflects a change in enzyme specificity toward a new, unidentified substrate.

Immunohistochemical analysis of human and baboon organs indicated that EPCR expression is quite specific to endothelial cells and that it is expressed primarily on the surface of large vessels.[14] In endothelial cell cultures of human, bovine, or murine origin, EPCR expression was down-regulated by the inflammatory mediator TNF-α.[10,15] Because protein C activation and function were shown to play a critical role in the host defense against bacterial challenge,[4,16,17] we thought it was important to understand the regulation of EPCR in vivo during challenges with endotoxin. During our analysis of the 5′ flanking region of the murine and human EPCR genes, we identified a thrombin response element, CCCACCCC, that in the context of the rest of the EPCR promoter, was necessary for the induction of murine EPCR mRNA by thrombin in cell culture.[18] The presence of a positive regulatory element in the gene raised the question of whether thrombin might overcome down-regulation by TNF-α in vivo.

From the Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma; Pharmaceuticals Development Research Laboratories, Teijin, Ltd, Tokyo, Japan; Howard Hughes Medical Institute, Oklahoma City, Oklahoma; and the Departments of Pathology and Biochemistry and Molecular Biology, University of Oklahoma Health Science Center, Oklahoma City, Oklahoma.

Submitted September 13, 1999; accepted November 12, 1999.

Supported by a grant awarded by the National Heart, Lung and Blood Institute of the National Institutes of Health (grant PO1 HL 54804) to C.T.E.

Reprints: Charles T. Esmon, Cardiovascular Biology Research, Oklahoma Medical Research Foundation, 825 NE 13th Street, Oklahoma City, OK 73104; e-mail: charles-esmon@omrf.ouhsc.edu.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

and heart was similar. Immunohistochemical analysis of the tissues before and after endotoxin exposure indicated that EPCR expression was still restricted to the large vessels and was low to absent in the capillaries (data not shown), as was found previously in tissue from a patient who died of bronchopulmonary dysplasia.[14] The increase in EPCR was temporally linked to fibrinogen consumption, which is indicative of thrombin generation. Therefore, we considered the possibility that thrombin might be responsible for up-regulation of the EPCR gene.

Thrombin generation contributes to induction of rat EPCR mRNA

To examine the possible role of thrombin in endotoxin-mediated up-regulation of EPCR mRNA, we infused hirudin, a specific thrombin inhibitor, before administration of LPS. As expected, control experiments in which hirudin alone was infused did not result in changes in either EPCR mRNA or fibrinogen levels (data not shown). Hirudin diminished the LPS-mediated EPCR mRNA up-regulation in lung tissue (Figure 4A), decreasing the response from a 2.9-fold increase to a 1.4-fold increase ($P = .0022$) (Figure 4B). Similar results were obtained in heart tissue. The fibrinogen level decreased less in the presence of hirudin than in its absence, but there was still a decrease (Figure 4C), indicating either that the hirudin had not totally inhibited thrombin or that other factors contributed to the decreases in fibrinogen. These results implicate thrombin as a major mediator of endotoxin-induced up-regulation of EPCR mRNA.

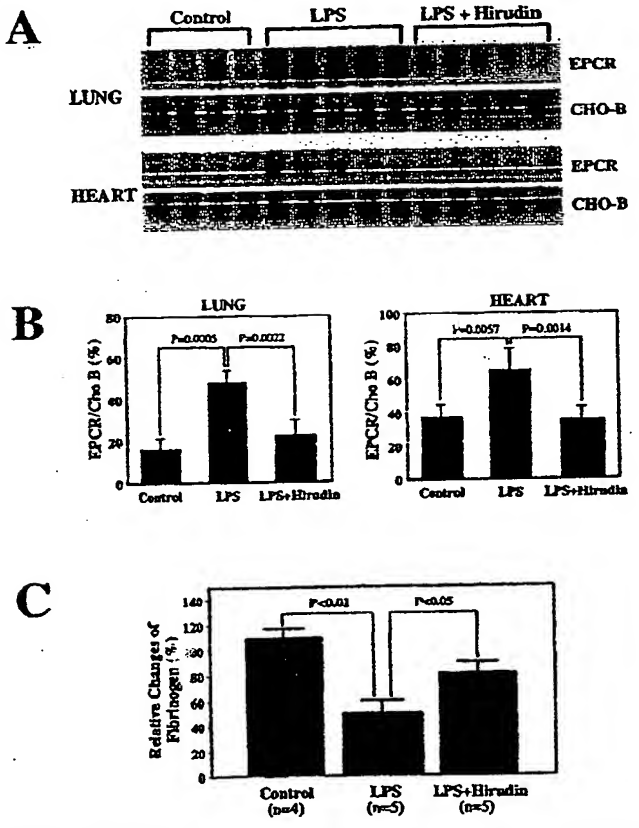


Figure 4. Decrease by hirudin administration of LPS-mediated up-regulation of EPCR mRNA levels. (A) Rats were surgically prepared and injected with saline (N) or LPS alone (LPS) or infused with hirudin before and after LPS injection (LPS + hirudin). Total RNA was prepared from rat lungs and hearts isolated 6 hours after LPS injection and used for Northern blot analysis. (B) Below the Northern blot is the quantitation of the change based on the ratio of the intensity of EPCR to CHO-B mRNA on the PhosphorImager. (C) Changes in fibrinogen levels in the above animals treated with LPS or LPS plus hirudin.

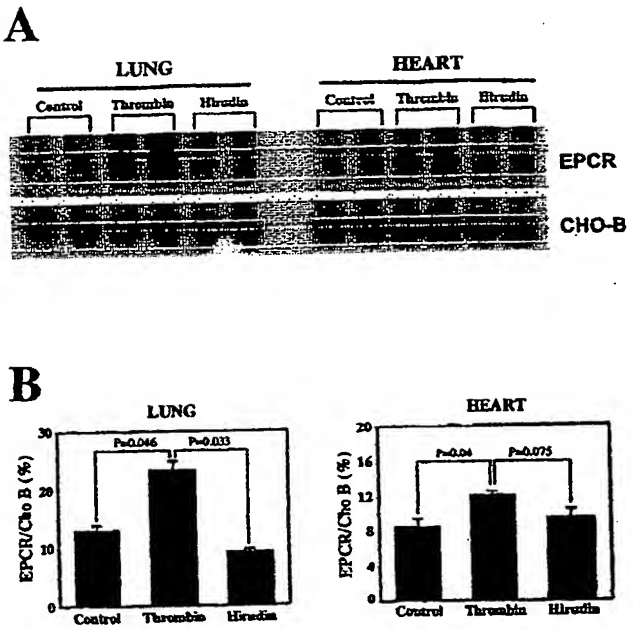


Figure 5. Increases in EPCR mRNA levels in rat lungs with infusion of thrombin. Bovine thrombin (5 U/kg infused per hour) and hirudin (100 U/kg infused per hour) were infused into the femoral vein of rats, and the lungs and hearts were removed 3 hours later for the extraction of total RNA. (A) Northern blot analysis. (B) Below the Northern blot is the quantitation of the change based on normalization of the signal of EPCR mRNA to that of CHO-B mRNA on the PhosphorImager.

Thrombin alone can up-regulate EPCR expression in vivo

To test directly the possibility that thrombin can up-regulate EPCR mRNA, thrombin was infused into rats for 3 hours at the rate of 5 U/kg per hour. EPCR mRNA levels had risen approximately 2-fold by the end of this infusion (Figure 5). Hirudin was used as a control protein in the infusion; there was no change in EPCR mRNA levels in response to hirudin infusion alone.

Endotoxin-induced increases in soluble EPCR levels in rats can be blocked by hirudin

In addition to increasing the endotoxin up-regulation of EPCR mRNA, endotoxin injection increased soluble EPCR antigen levels in serum (approximately 4-fold after 6 hours) (Figure 6A). Like the mRNA elevation, this rise in serum EPCR levels was also blocked by hirudin (Figure 6A). These results implicate thrombin as a major mediator of endotoxin-induced shedding of soluble EPCR. To further test this hypothesis, thrombin was infused for 3 hours at the rate of 5 U/kg per hour. At the end of this infusion, the serum EPCR levels had risen approximately 2-fold (Figure 6B) compared with the control and hirudin-only groups.

We next examined the time course of soluble EPCR release. Because most rats die within 24 hours when injected with an LD95 dose (50 mg/kg) of endotoxin, we reduced the dose to 20 mg/kg. Serum was collected through the tail vein at different times. Even this lower dose caused 2 of the 4 rats to die within 24 hours. In Figure 6C, therefore, each animal is represented individually, with a different symbol, in the time course. The last point represents serum collected at the time of death except for the 2 animals that survived for 24 hours. The soluble EPCR levels rose with time. In the surviving animals, the levels returned toward baseline values by 24 hours.

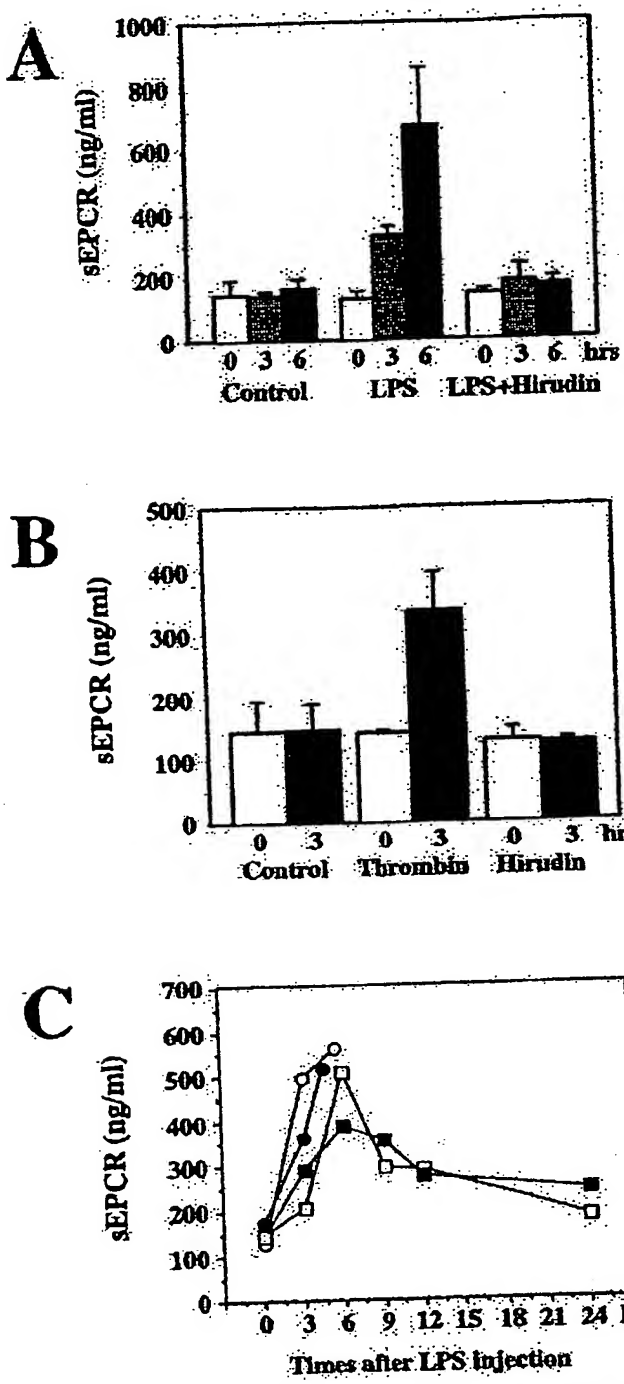


Figure 6. Increases by LPS in the soluble EPCR levels in rats that can be blocked by hirudin. (A) Serum from normal controls (n = 6), rats given LPS (n = 4), and rats given LPS plus hirudin (n = 6) at 0, 3, and 6 hours after administration of the agents were assayed for soluble EPCR levels by enzyme-linked immunosorbent assay (ELISA). (B) Changes in soluble EPCR levels in rats infused with bovine thrombin or hirudin alone. (C) Rats were injected with LPS (20 mg/kg) through the tail vein. Serum samples were collected at the indicated times through the tail vein and were assayed for the soluble EPCR by ELISA. In the 2 animals that died, the last data point corresponds to the serum sample obtained at the time of death.

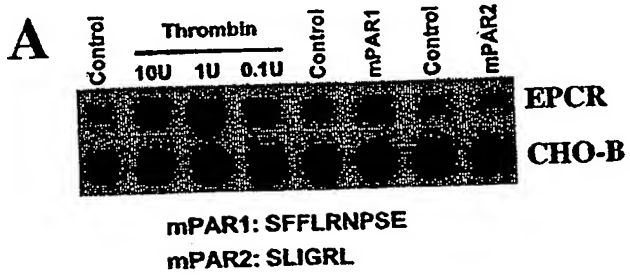
Thrombin and mPAR1 can up-regulate EPCR mRNA levels in cell culture

The in vivo thrombin response could be indirect. To determine whether thrombin affected expression of EPCR in rat aortic endothelial cells directly in culture, these cells were treated with thrombin. After a 6-hour incubation with even relatively low concentrations of thrombin (0.1 U/mL), EPCR mRNA levels were increased (Figure 6A). Thrombin increased EPCR mRNA in a time-dependent manner, with peak levels reached at 12 hours of incubation (data not shown).

To test whether thrombin-mediated up-regulation of EPCR is mediated by a protease-activated receptor, we stimulated the cells with the agonist peptide SFFLRNPSE for mPAR1[26] or SLIGRL for mPAR2.[27] After a 6-hour incubation, the mPAR1 agonist peptide increased EPCR mRNA levels approximately 2-fold (Figure 7), a response similar to that observed with thrombin. Incubation with mPAR2 had no effect on EPCR mRNA levels. These results indicate that thrombin mediates up-regulation of EPCR mRNA in endothelial cells through mPAR1.

Endotoxin does not change tissue levels of EPCR

Given that endotoxin elicits both an increase in EPCR mRNA and soluble EPCR levels, the question of whether there is a net change in tissue EPCR expression arises. We found that EPCR levels in the heart and lung changed very little in response to either endotoxin or thrombin infusion (Table 1). These results suggest that the increase in mRNA may prevent the shedding of the receptor from diminishing EPCR levels in the endothelium.



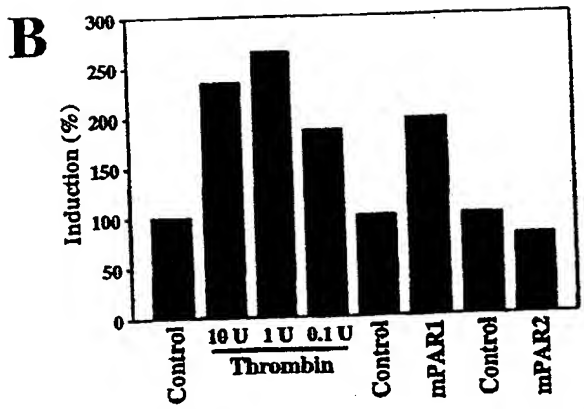


Figure 7. Increases in EPCR mRNA levels in endothelial cells in culture by thrombin and murine protease-activated receptor 1. Rat aortic endothelial cells were maintained in serum-free media for 24 hours and were then treated with 0.1, 1, or 10 U/mL of bovine thrombin, 10 μmol/L of murine protease-activated receptor (mPAR) 1 peptide, and 10 μmol/L of mPAR2 peptide for 6 hours. (A) Total RNA was extracted and 15 μg of the RNA was analyzed by Northern blotting and compared with CHO-B mRNA levels. (B) The changes in the mRNA levels were quantitated with a PhosphorImager on the basis of the increase in the ratio of the EPCR intensity to the CHO-B intensity.

Table 1. Endothelial cell protein C receptor (EPCR) levels in tissue from control rats and from rats given lipopolysaccharide B from E coli O127:B8 (LPS) or thrombin

Tissue	Control (n = 5)	LPS (n = 5)	Thrombin (n = 2)
Lung	904.1 ± 284.5	675.2 ± 255.4	984.7 ± 263.1
Heart	1324.7 ± 278.1	1593.6 ± 645.2	1290.6 ± 312.9

Rats were either injected with endotoxin (50 mg/kg) or infused with thrombin (5 U/kg per hour for 3 hours). Six hours after endotoxin injection and 3 hours after thrombin infusion, they were perfused transcardially and lung and heart tissue were extracted with use of Triton X-100. EPCR antigen in the tissues was measured by an enzyme-linked immunosorbent assay. In controls, the results are expressed as the mean ± SE. In rats given LPS or the thrombin infusion, the results are expressed as the mean ± SD.

EPCR levels are given in mg/g.

Vascular distribution after endotoxin challenge

One possible cause of EPCR mRNA up-regulation is that EPCR expression is selectively increased in the microvasculature. Immunohistochemical staining, performed essentially as described previously,[14] failed to detect increases in EPCR expression in the microvasculature. In addition, the EPCR expression levels in the kidney and brain did not appear to change significantly, which constitutes strong evidence against the possibility of organ-specific differences in regulation (data not shown).

Discussion

These studies demonstrate that, contrary to predictions derived from in vitro cell-culture studies, the in vivo response to LPS is to up-regulate EPCR mRNA levels. Presumably, this is due to increases in transcription, since a putative thrombin response element is present in the 5′ region of the EPCR gene, expression of luciferase reporter constructs containing this element can be enhanced by thrombin treatment of the transfected cells,[18] and transgenic animals with this region of the EPCR promoter have increased expression of the transgene only if the thrombin response element is not mutated (Gu and Esmon, unpublished observations). Our findings with hirudin indicate that the endotoxin-mediated up-regulation has a strong requirement for thrombin generation. This could result from either thrombin effects on the endothelium or the generation of thrombin-dependent products, which could include, for instance, platelet-release products. On the basis of the cell-culture data, which showed that thrombin could increase EPCR mRNA directly, it appears that at least some of the stimulation is due to direct thrombin effects on endothelium. This is at least partly mediated by mPAR1 because mPAR1 agonist peptide and thrombin induced a similar enhancement of EPCR mRNA. Thus, it is likely that the thrombin-induced increase in EPCR mRNA serves to up-regulate EPCR protein synthesis. This increased synthesis may be offset by increased shedding of the receptor, as reflected by the substantial increases in serum EPCR levels observed in response to endotoxin.

Studies have shown that thrombin or the PAR1 agonist peptide can enhance EPCR shedding from human umbilical vein endothelial cells.[28] This is apparently mediated by the activation of a metalloproteinase. It is unlikely that the soluble EPCR that appeared after endotoxin treatment resulted from endothelial cell death, since hirudin, which did not protect the animals from the lethal effects of endotoxin, blocked the endotoxin-induced soluble EPCR formation. It is also unlikely that the soluble EPCR was caused by alternative splicing of the mRNA. Although we have detected alternatively spliced mRNA that would code for soluble EPCR, the levels of the alternatively spliced transcript were low both before and after endotoxin treatment (unpublished observations).

Other agonists can also elicit EPCR shedding, raising the question of which agonists are most important in vivo. Our current data indicate that thrombin appears to be critical for shedding, at least in the context of endotoxin. We previously found, however, that other agonists can work synergistically with thrombin to augment shedding from endothelium in culture.[28] The possibility that thrombin works synergistically with other agonists to increase EPCR shedding further cannot be addressed readily by in vivo studies.

Our original goal in searching for EPCR was to attempt to explain the ability of APC to modulate the response to lethal levels of *E coli* in baboons[4] and to protect against meningococcemia in humans.[5-7] When EPCR mRNA and function were found to be down-regulated in response to endotoxin and TNF-α in cell culture, it appeared that EPCR might not be a candidate for the in vivo response, at least in meningococcemia. Patients with meningococcemia are often treated relatively late in the disease course— many hours after the onset of the infection, when the cell-culture data would predict that EPCR levels would be extremely low. The observations presented here suggest that EPCR would be relatively well expressed under these conditions. Consistent with this possibility, immunohistochemical studies of vessels from a patient who died of respiratory distress syndrome revealed high levels of EPCR expression.[14] On the basis of these findings and the observation that the macromolecular specificity of APC is altered when bound to soluble EPCR,[13] the possibility that this receptor is involved in modulating the inflammatory responses in gram-negative sepsis remains viable. Initial experiments in a primate model of *E coli* septic shock have provided preliminary support for this idea.[24] In these experiments, blocking protein C binding to EPCR with a monoclonal antibody dramatically increased leukocyte migration into the tissues and induced a capillary leak syndrome in response to low-level *E coli* infusion.

In studies with results possibly related to the current findings, we identified moderately high levels of EPCR in the plasma of healthy human donors.[29] The levels of plasma EPCR were elevated in inflammatory disease states such as sepsis and lupus erythematosus.[30] It is possible that the induction of mRNA observed here is a mechanism to help compensate for the loss of EPCR from the membrane surface that occurs in disease states associated with coagulation and inflammation. The observation that the shedding of EPCR and the increase in mRNA are temporally similar and both mediated by thrombin suggests that the release of soluble EPCR may play an important, unidentified role in the physiologic response to endotoxin. In support of this conclusion, we observed specific binding sites for soluble EPCR on activated leukocytes[31] and found that the binding interaction is mediated largely by proteinase 3, the autoantigen found in Wegener's granulomatosis. Alternatively, although the levels of soluble EPCR achieved in this study were too low to impair APC anticoagulant function, most of the soluble EPCR would be in complex with the protein C-APC, since the concentrations of these components are higher than the Kd for EPCR-protein C interaction (≈30-50 nmol/L). Therefore, if the complex does have altered specificities, increases in soluble EPCR would allow expression of these activities throughout the blood stream.

Acknowledgments

We thank Jeff Box for assistance with the figures, Jeff Mollica for performing the plasma APC assays, Zoltan Laszik for performing the immunohistochemical studies, and Nici Barnard for preparing the final manuscript.

References

1. Esmon CT. The roles of protein C and thrombomodulin in the regulation of blood coagulation. J Biol Chem. 1989;264:4743-4746.
2. Seligsohn U, Berger A, Abend M, et al. Homozygous protein C deficiency manifested by massive thrombosis in the newborn. New Engl J Med. 1984;310:559-562.
3. Dreyfus M, Magny JF, Bridey F, et al. Treatment of homozygous protein C deficiency and neonatal purpura fulminans with a purified protein C concentrate. New Engl J Med. 1991;325:1565-1568.
4. Taylor FB Jr, Chang A, Esmon CT, D'Angelo A, Vigano-D'Angelo S, Blick KE. Protein C prevents the coagulopathic and lethal effects of *E. coli* infusion in the baboon. J Clin Invest. 1987;79:918-925.
5. Smith OP, White B, Rafferty M. Successful treatment of meningococcal induced protein C deficiency/purpura fulminans in children with protein C concentrate and heparin [abstract]. Thromb Haemost. 1997;419(suppl). Abstract PS-1709.
6. Esmon CT, Schwarz HP. An update on clinical and basic aspects of the protein C anticoagulant pathway. Trends Cardiovasc Med. 1995;5:141-148.
7. Rivard GE, David M, Farrell C, Schwarz HP. Treatment of purpura fulminans in meningococcemia with protein C concentrate. J Pediatr. 1995;126:646-652.
8. Grey ST, Tsuchida A, Hau H, Orthner CL, Salem HH, Hancock WW. Selective inhibitory effects of the anticoagulant activated protein C on the responses of human mononuclear phagocytes to LPS, IFN-gamma, or phorbol ester. J Immunol. 1994;153:3664-3672.
9. Grinnell BW, Hermann RB, Yan SB. Human protein C inhibits selectin-mediated cell adhesion: role of unique fucosylated oligosaccharide. Glycobiology. 1994;4:221-226.
10. Fukudome K, Esmon CT. Identification, cloning and regulation of a novel endothelial cell protein C/activated protein C receptor. J Biol Chem. 1994;269:26,486-26,491.
11. Bangalore N, Drohan WN, Orthner CL. High affinity binding sites for activated protein C and protein C on cultured human umbilical vein endothelial cells independent of protein S and distinct from known ligands. Thromb Haemost. 1994;72:465-474.
12. Stearns-Kurosawa DJ, Kurosawa S, Mollica JS, Ferrell GL, Esmon CT. The endothelial cell protein C receptor augments protein C activation by the thrombin-thrombomodulin complex. Proc Natl Acad Sci U S A. 1996;93:10,212-10,216.
13. Regan LM, Stearns-Kurosawa DJ, Kurosawa S, Mollica J, Fukudome K, Esmon CT. The endothelial cell protein C receptor: inhibition of activated protein C anticoagulant function without modulation of reaction with proteinase inhibitors. J Biol Chem. 1996;271:17,499-17,503.
14. Laszik Z, Mitro A, Taylor FB Jr, Ferrell G, Esmon CT. Human protein C receptor is present primarily on endothelium of large blood vessels: implications for the control of the protein C pathway. Circulation. 1997;96:3633-3640.
15. Fukudome K, Esmon CT. Molecular cloning and expression of murine and bovine endothelial cell protein C/activated protein C receptor (EPCR). The structural and functional conservation in human, bovine, and murine EPCR. J Biol Chem. 1995;270:5571-5577.
16. Taylor F, Chang A, Ferrell G, et al. C4b-binding protein exacerbates the host response to *Escherichia coli*. Blood. 1991;78:357-363.
17. Hancock WW, Tsuchida A, Hau H, Thomson NM, Salem HH. The anticoagulants protein C and protein S display potent anti-inflammatory and immunosuppressive effects relevant to transplant biology and therapy. Transplant Proc. 1992;24:2302-2303.
18. Gu JM, Esmon CT. Functional characterization of 5'-flanking region of the mouse endothelial protein C receptor (EPCR) gene [abstract]. Mol Biol Cell. 1997;8(suppl):228a.
19. Owen WG, Esmon CT, Jackson CM. The conversion of prothrombin to thrombin: I: characterization of the reaction products formed during the activation of bovine prothrombin. J Biol Chem. 1974;249:594-605.
20. Diglio CA, Liu W, Grammas P, Giacomelli F, Wiener J. Isolation and characterization of cerebral resistance vessel endothelium in culture. Tissue Cell. 1993;25:833-846.
21. Hyman R, Lesley J, Schulte R. Somatic cell mutants distinguish CD44 expression and hyaluronic acid binding. Immunogenetics. 1991;33:392-395.
22. Yao L, Pan J, Setiadi H, Patel KD, McEver RP. Interleukin 4 or oncostatin M induces a prolonged increase in P-selectin mRNA and protein in human endothelial cells. J Exp Med. 1996;184:81-92.
23. Fukudome K, Kurosawa S, Stearns-Kurosawa DJ, He X, Rezaie AR, Esmon CT. The endothelial cell protein C receptor: cell surface expression and direct ligand binding by the soluble receptor. J Biol Chem. 1996;271:17,491-17,498.
24. Taylor FB Jr, Stearns-Kurosawa DJ, Kurosawa S, et al. The endothelial cell protein C receptor aids in host defense against *Escherichia coli* sepsis. Blood. In press.
25. Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from the lethal effect of endotoxin. Science. 1985;229:869-871.
26. Kahn ML, Zheng Y-W, Huang W, et al. A dual thrombin receptor system for platelet activation. Nature. 1998;394:690-694.
27. Nystedt S, Larrsson AK, Aberg H, Sundelin J. The mouse proteinase-activated receptor-2 cDNA and gene. Molecular cloning and function expression. J Biol Chem. 1995;270:5950-5955.
28. Xu J, Qu D, Esmon NL, Esmon CT. Metalloproteolytic release of endothelial cell protein C receptor. J Biol Chem. In press.
29. Kurosawa S, Stearns-Kurosawa DJ, Hidari N, Esmon CT. Identification of functional endothelial protein C receptor in human plasma. J Clin Invest. 1997;100:411-418.
30. Kurosawa S, Stearns-Kurosawa DJ, Carson CW, D'Angelo A, Della Valle P, Esmon CT. Plasma levels of endothelial cell protein C receptor are elevated in patients with sepsis and systemic lupus erythematosus: lack of correlation with thrombomodulin suggests involvement of different pathological processes. Blood. 1998;91:725-727.
31. Stearns-Kurosawa DJ, Esmon CT, Kurosawa S. The endothelial protein C receptor, neutrophil, proteinase-3 and Wegener's granulomatosis [abstract]. Thromb Haemost. Aug 1999(suppl);215.

Haematologica 1999; 84:452-460 trends in hematology

Antithrombin replacement in patients with sepsis and septic shock

DANIELA GIUDICI,* FRANCESCO BAUDO,# GUALTIERO PALARETI,° ADRIANO RAVIZZA,^ LORENZA RIDOLFI,@ ARMANDO D'ANGELO§

*Unità di Terapia Intensiva e §Servizio di Coagulazione, IRCCS H S.Raffaele, Milan; #Unità di Emostasi e Trombosi e ^Dipartimento di Terapia Intensiva, Ospedale Niguarda, Milan; °Dipartimento di Angiologia e @Unità di Terapia Intensiva, Ospedale S. Orsola, Bologna, Italy

ABSTRACT

Sepsis is a frequent complication of critically ill patients and its incidence is increasing. Currently, septic shock is the most common cause of death in non-coronary intensive care units. Over the last 10 to 15 years, new antibiotics and increasingly sophisticated critical care have had little impact on the mortality rate of septic shock. The italian SESPIS Study, carried out in 99 intensive care units in 1994, reported mortality rates of 52% and 82% for severe sepsis and septic shock respectively. New therapeutic approaches aimed at neutralizing microbial toxins and modulating host mediators have shown some efficacy in large clinical trials and/or in animal models, but to date, no therapy of sepsis aimed at reversing the effects of bacterial toxins or of harmful endogenous mediators of inflammation has gained widespread clinical acceptance. Because of the strong association of severe sepsis with a state of activation of blood coagulation and the potential role of capillary thrombosis in the development of the multiple organ dysfunction syndrome, anticoagulant agents have been tested in the setting of spetic shock. However, neither administration of heparin nor of active site-blocked factor Xa or of anti-tissue factor antibodies has proven effective in preventing deaths due to septic shock in animal models. In contrast, infusion of antithrombin, protein C, or tissue factor pathway inhibitor all resulted in a significant survival advantage in animals receiving lethal doses of *E. Coli*. Antithrombin concentrates have been used in a significant number of critically ill patients. A double-blind, placebo controlled study carried out in 3 Italian intensive care units has recently shown that the administration of antithrombin aimed at normalizing plasma antithrombin activity had a net beneficial effect on 30-day survival of patients requiring respiratory and/or hemodynamic support because of severe sepsis and/or post-surgery complications.

Key words: sepsis, septic shock, diffuse intravascular fibrin formation, antithrombin replacement therapy, protein C

Correspondence: Armando D'Angelo, M.D., Servizio di Coagulazione, IRCCS H S. Raffaele, via Olgettina 60, 20132 Milan, Italy. Phone: international +39-02-26432228 - Fax: international +39-02-26432640 o E-mail: armando.dangelo@hsr.it

Infection is a common cause of admission into intensive care units (ICUs) and a frequent complication of critically ill patients. A series of factors have contributed to the increasing incidence of sepsis and of septic shock. Immunosuppressive therapy for malignancy, organ transplantation, or inflammatory disease places patients at increased risk of infectious complications. Patients predisposed by underlying diseases such as diabetes mellitus, renal failure, and cancer are more likely to suffer an increased rate of infections because they have now a longer life-expectation. Invasive life support procedures (hemodynamic and respiratory support) and broad-spectrum antibiotics have created a large hospital-based population at risk of nosocomial infection by resistant micro-organisms.

Septic shock is currently the most common cause of death in non-coronary ICU.[1,2] Mortality is related to the severity of sepsis and of the underlying disorder that is nearly always present. Agreement about the definition of a septic syndrome has been only recently achieved. In 1992, a Consensus Conference of the *American College of Chest Physicians and Society of Critical Care Medicine* established a set of definitions that could be applied to patients with sepsis and its sequelae.[3] The term *sepsis* implies a clinical response arising from infection, but a similar, or even identical, response may also develop in the absence of infection. This systemic inflammatory response syndrome (SIRS) can occur following a wide variety of insults, infectious or non-infectious, the latter including pancreatitis, ischemia, multiple trauma and tissue injury, etc. SIRS is defined by the occurrence of two or more of the following conditions: temperature >38°C or <36°C, heart rate >90 beat/min, respiratory rate >20 breath/min or PaCO2 < 32 torr, white blood cell count >12,000/μL or >10% immature forms. When the systemic inflammatory response syndrome is the result of a confirmed infectious process, it is termed sepsis.

Sepsis and its sequelae represent a continuum of clinical and pathophysiological severity. Sepsis is defined as *severe* when it is associated with organ dysfunction, hypoperfusion abnormalities or sepsis-

induced hypotension. Hypoperfusion abnormalities include lactic acidosis, oliguria, or an acute alteration of mental status. Sepsis-induced hypotension is defined by the presence of a systolic blood pressure of <90 mm Hg or its reduction by more than 40 mm Hg from the baseline, in absence of other causes for hypotension (cardiogenic shock etc.). *Septic shock* is a subset of severe sepsis and is defined as sepsis-induced hypotension, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction. Patients receiving inotropic or vasopressor agents might no longer be hypotensive by the time they manifest hypoperfusion abnormalities or organ dysfunction, yet they are still considered affected by septic shock.

A frequent complication of SIRS is the development of organ system dysfunction, as a process of progressive failure of several interdependent organ systems. The detection of altered organ function in acutely ill patients constitutes a syndrome that should be termed *multiple organ system dysfunction syndrome* (MODS), in which organ function is not capable of maintaining homeostasis.[4,5] The term *dysfunction* emphasizes the dynamic nature of the process, although specific descriptions of this continuous process are not currently available. Multiple organ dysfunction syndrome is subject to modulation by numerous factors, both interventional and host-related, at varying time periods. MODS may be primary when it occurs early in response to a well-defined insult such as trauma, pulmonary contusion, rhabdomyolysis, massive transfusions, or it may be secondary to the host response to the insult when it is characterized by a generalized activation of the inflammatory reaction in organs remote from the initial insult. When due to infection, secondary MODS usually evolves after a latent period after the provoking injury or event, and is a frequent complication of severe infection.

Pathogenesis

The pathogenesis of sepsis and septic shock are not completely understood. Gram-positive organisms releasing exotoxins, Gram-negative organisms containing endotoxins and fungi can initiate this pathogenic cascade. The process begins with the proliferation of micro-organisms at a nidus of infection. The organisms may invade the bloodstream directly (leading to a positive blood culture) or may proliferate locally and release various substances into the bloodstream. These events trigger host cells (neutrophils, monocyte-macrophages) to release a variety of interacting cytokines (tumor necrosis factor [TNF], interleukins, interferons). This results in the activation of several pathways (complement, coagulation, fibrinolytic, and hormonal) and in the increased production of numerous endogenous mediators (C5a, eicosanoids, endorphins, toxic oxygen radicals, nitric oxide, and platelet-activating factor), with profound physiologic effects on the cardiovascular system and on the function of other organs.[6]

Early in severe sepsis, systemic vascular resistance decreases – primarily mediated by the release of bradykinin and histamine – and cardiac output increases. In this hyperdynamic phase, septic shock is a classic form of distributive shock, resulting from abnormal distribution of blood flow. Despite an often elevated cardiac output, tissue oxygen utilization is reduced.[7,8] The decreased artero-venous oxygen difference suggests that oxygen is not reaching or not being used by tissues. The exact mechanisms responsible for decreased tissue perfusion are poorly understood. In septic shock, many vascular beds are dilated, but some are constricted, and some are occluded by microthrombi. The aggregation of neutrophils and platelets may lead to impairment of blood flow. Neutrophil migration occurs along the vascular endothelium, resulting in the release of many mediators and the migration of neutrophils into tissues. Neutrophils can release active oxygen species, such as superoxide radicals, that can directly damage cells. Components of the complement system, such as C5a, are activated. Inflammatory mediators, such as prostaglandins and leukotrienes are released from many types of cells and can cause either vasoconstriction or vasodilatation, with increased permeability of the vascular endothelium and passage of fluid from the intravascular to the interstitial fluid space. Endothelial damage may *per se* decrease oxygen and substrate utilization by the tissues.

In the hyperdynamic phase blood pressure is normal or slightly reduced, the skin is warm and dry, there is tachycardia, urine output is satisfactory, and the patient hyperventilates and is pyretic. Fever results from the direct effects of endotoxins and interleukin-1 on the hypothalamus. The release of inflammatory mediators and endothelial damage also lead to the development of diffuse intravascular fibrin formation (DIFF) and deposition, followed by a secondary bleeding tendency. DIFF decreases organ blood flow, causing hypoxia, lactic acidosis, organ dysfunction and failure.[9] This occurs especially in the circulation of the lungs, liver, kidneys, and gastric mucosa with the manifestations of secondary MODS.

If the clinical state is not recognized and treated within a few hours the patient enters the hypotensive phase of septic shock, in which the combination of decreased systemic vascular resistance and myocardial depression induces hypotension which is independent from adequate fluid resuscitation.[10] A reversible depression of myocardial function, with decreased ejection fraction and left ventricular dilation, is common in septic shock. Circulating antiinotropic substances, termed myocardial depressant substances,[11,12] may play an important role in the pathogenesis of myocardial depression. In the hypotensive phase the patient is oliguric with cold, pale skin, and is cyanotic, features which are typical of an established shock syndrome. As a consequence of the arteriolar dilation and of the increased capillary and

post-capillary venule permeability, especially in the infected tissues, there is increased fluid transfer from capillaries to the interstitial fluid. The hypovolemia decreases venous return, cardiac output and blood pressure. Baroreceptor compensation increases sympathetic activity so causing vasoconstriction in the skin, the splanchnic areas, kidney and muscles.

Bronchoconstriction is an early finding in many patients with severe sepsis. This is probably due to endotoxin or to release of inflammatory mediators. At this time the chest radiograph is often normal, but gas exchange may be mildly abnormal. Later, if septic shock occurs many patients develop diffuse alveolar damage consistent with the *adult respiratory distress syndrome*[13] (ARDS). From 40% to 60% of patients with Gram-negative septic shock develop ARDS.[14] Alveolar-capillary membrane damage allows for leakage of fluid and proteins into the pulmonary interstitium. Alveoli are subsequently flooded, causing a marked increase in intrapulmonary shunting and severe arterial hypoxia. At this stage the chest radiograph demonstrates diffuse bilateral alveolar infiltrates. Hypoxic pulmonary vasoconstriction, *in situ* thrombosis, and aggregation of neutrophils and platelets in the pulmonary microvascular system increase pulmonary artery pressure and right ventricular afterload, leading to a worsening of right ventricular performance.

In addition to the cardiopulmonary systems, other systems may sequentially become dysfunctional in septic shock because of the role of inflammatory mediators. Visceral hypoperfusion and decreased intestinal peristalsis may lead to alterations of the barrier function of the gastrointestinal tract; gastrointestinal bleeding may follow stress ulceration of the gastric mucosa. Liver dysfunction may manifest as hyperbilirubinemia, elevated aminotransferase levels, cholestasis, progressive and intractable hypoglycemia and hypoalbuminemia. As kidney function declines, urine output falls and blood urea and creatinine levels rise. Renal failure is mainly due to acute tubular necrosis induced by hypotension or capillary injury, but drug-induced renal damage may also occur. Alterations of the mental status can occur, ranging from mild confusion and lethargy, to stupor and coma; abnormalities in the blood brain barrier and changes in the concentrations of circulating aminoacids frequently accompany this *obtundation of sepsis*.[15] Abnormalities of the clotting system, ranging from mild prolongation of the prothrombin time and of the partial thromboplastin time, to profound thrombocytopenia and frank disseminated intravascular coagulation are common in patients with septic shock.

Treatment

The treatment strategy for severe sepsis and septic shock is based on the provision of intensive life supports, the eradication of micro-organisms, the neutralization of microbial toxins, and the modulation of host mediators.[16]

Intensive life supports to maintain vital functions involve careful monitoring of patients in a critical care unit setting. Metabolic derangements (electrolyte disturbances, acidosis) should be aggressively corrected, as they can worsen the hemodynamic abnormalities of septic shock. The hematocrit should be maintained above 30% to improve the oxygen-carrying capacity. Respiratory failure requires mechanical ventilation.

Patient monitoring is essential to the choice of the cardiovascular support and includes cardiac rhythm monitoring, intra-arterial invasive blood pressure monitoring, right-sided heart catheterization with a Swan-Ganz catheter, and laboratory monitoring of the metabolic profile.

All patients with severe sepsis and septic shock have moderate to profound intra-vascular hypovolemia due to vasodilatation and loss of fluids in the extravascular spaces. The type and amount of fluid (crystalloids, colloids, and albumin) are highly controversial. When the mean arterial pressure is less than 60 mmHg, volume resuscitation is the initial treatment of choice, to avoid limiting coronary and cerebral artery autoregulation and to prevent inadequate tissue perfusion. Fluids should be infused rapidly to maximize ventricular performance. In general, this can be obtained at a pulmonary capillary wedge pressure of 12 to 15 mm Hg. Patients with higher wedge pressures carry a substantial risk of developing pulmonary edema. If, in spite of volume resuscitation, the mean arterial pressure remains below 60 mmHg when the pulmonary capillary wedge pressure is above 15 mmHg, inotropic agents used singly or in combination may offset the myocardial dysfunction and augment cardiac output. No universal agreement exists as to how these agents should be utilized, in view of their different effects on cardiac stimulation, vasoconstriction and vasodilatation (Table 1). Dopamine is commonly employed in this setting because of the β-adrenergic effects enhancing cardiac performance and the α-adrenergic effects supporting arterial blood pressure. The potent vasoconstrictor effects of norepinephrine are advantageous in septic shock patients who are unresponsive to high doses of dopamine. Dobutamine may be used alone or in combination with other catecholamines to improve cardiac performance.

Eradication of micro-organisms requires early antibiotic administration. This is initially empirical, using broad-spectrum antibiotics against Gram-positive and Gram-negative bacteria and sometimes against fungi. Cultures of body fluids are helpful in the identification of the micro-organisms involved, but radiological investigations may be required to discover the site of infection. Specific foci of infection should be drained and necrotic tissue surgically removed when appropriate.

Septic shock may, however, present with no identifiable source of infection and with negative blood

Table 1. Vasopressor therapy in septic shock.

Inotropic agent	Cardiac stimulation (β-1)	Vaso-constriction (α-1)	Vaso-dilatation (β-2)
Dopamine 5-10 μg/kg/min	++	+	++
↓			
Dopamine 10-20 μg/kg/min	+++	+++	+
↓			
Norepinephrine 0.02-0.2 μg/kg/min	+++	++++	0
+ Dopamine 2-4 μg/kg/min	++	+	++
↓			
Norepinephrine 0.02-0.2 μg/kg/min			
+ Dopamine 2-4 μg/kg/min	++	+	++
+ Dobutamine 5-10 μg/kg/min	++++	+	++

cultures especially in neutropenic patients.

Over the last 10 to 15 years, new antibiotics and increasingly sophisticated critical care have had little impact on the mortality rate of septic shock, which remains extremely high as demonstrated by the results of the Italian SEPSIS Study.[17] The aim of this prospective, multicenter investigation was to evaluate the clinical outcome of consecutive patients admitted to intensive care units on the basis of the diagnostic criteria of the ACCP/SCCM Consensus Conference.[3] The study was carried out in 99 ICUs in Italy from April 1993 to March 1994. In a preliminary analysis of 1100 patients, severe sepsis and septic shock had mortality rates of 52.2% and 81.8% respectively (Table 2). As a result, new therapeutic approaches have been tested, aimed at neutralizing microbial toxins and modulating host mediators (Table 3). Some of the agents have shown some efficacy in large multicenter clinical trials (anti-endotoxin monoclonal antibodies,[18-19]), others only in animal models (monoclonal anti-TNF antibodies,[20-22]). To date however, no therapy of sepsis aimed at reversing the effects of bacterial toxins or of harmful endogenous mediators has gained widespread clinical acceptance.

Antithrombin concentrates in sepsis and septic shock

In view of the strong association of severe sepsis with a state of activation of blood coagulation and the potential role of capillary thrombosis in the development of MODS, anticoagulant agents have been tested in the setting of septic shock. However, neither administration of heparin[23] nor of active site-blocked factor Xa[24] have proven effective in preventing deaths due to septic shock in animal models. Even the administration of anti-tissue factor antibodies did not prevent severe manifestations of septic shock in animal models, although resulting in effective blockade of the clotting system.[25] In contrast, infusion of natural inhibitors of blood coagulation (antithrombin, protein C, tissue factor pathway inhibitor), all

Table 2. Mortality rate of consecutive patients admitted to Italian intensive care units: results of the Italian SEPSIS Study.

ACCP/SCCM diagnosis on admission:		Nil	SIRS	Sepsis	Severe sepsis	Septic shock
Patients	n.	421	573	50	23	33
	(%)	38.3	52.1	4.5	2.1	3.0
Mortality rate	(%)	24.0	26.5	36.0	52.2	81.8

Table 3. Novel therapeutic approaches in septic shock.

Neutralization of microbial toxins	Modulation of host mediators
Anti-endotoxin antibodies:	Anti-TNF antibodies (TNF MoAb)
Polyclonal antibodies (*E.coli* J5 antiserum, antibodies to Lipid A)	Interleukin-1 receptor antagonists
Monoclonal antibodies (HA-1A, E5)	Anti-C5a antibodies
Lipid A analogs (lipid X, monophosphoryl lipid A)	Eicosanoid inhibitors
	Antioxidants
Cationic polypeptide antibiotics	Corticosteroids
	PAF antagonists
Plasma detoxification (plasmapheresis, continuous artero-venous hemofiltration)	Inhibitors of coagulation (antithrombin, protein C)

resulted in a significant survival advantage in animals receiving lethal doses of *E. coli*.[26-29]

Because of their commercial availability, antithrombin concentrates have been used in a significant number of critically ill patients. Antithrombin (AT), a glycoprotein synthesized by the liver and the kidney, is a main physiologic inhibitor of serine proteases generated during blood coagulation (FIIa, FIXa, FXa, FXIa, FXIIa).[30] The rate of neutralization of the above-mentioned proteases is increased by 3 orders of magnitude in the presence of heparin and heparin-sulphate. The concentration of AT in plasma is decreased in conditions associated with diffuse intravascular fibrin formation, particularly in sepsis and shock.[31-34] The decreased plasma concentration of AT may be an indication of the role of DIFF in the pathogenesis of multi-organ failure; it is a poor prognostic factor and correlates with survival.[35-39] Two randomized studies addressed the use of AT concentrate supplementation in the treatment of severe sepsis and shock, but they did not include a placebo-control group. Blauhut *et al.* randomized 51 patients with shock of different etiology (sepsis, trauma, hepatic coma) to receive AT, heparin or AT + heparin.[34] The time to normalization of the platelet count and of the fibrinogen concentration was shorter in patients receiving AT, but no difference in survival was observed. In a subsequent study, including only

patients with traumatic shock, Vinazzer reported a significant reduction in the mortality of patients treated with AT concentrate.[40] However, in an Italian study of patients with an established diagnosis of DIFF, the administration of AT concentrates did not result in any significant survival advantage.[41] These results are difficult to interpret. In critically ill patients, the evaluation of the efficacy of therapeutic agents requires a double-blind design, to avoid the bias of the attending clinician who is confronted with patients with a potentially fatal outcome.[42] Fourrier *et al.* published the first randomized double blind, placebo-controlled study in septic shock. Patients treated with AT tended to have a survival advantage, but the difference from the placebo group did not reach conventional statistical significance.[43] Similar results were obtained by Lamy *et al.*[44] (Table 4).

We planned a double blind study to evaluate the effect of AT administration on survival of a selected group of patients requiring hemodynamic and/or respiratory support because of severe sepsis and/or post-operative complications.[47] A major assumption was that the observation of decreased AT levels – unrelated to evidence of impaired liver synthesis of the protein – may reflect uncontrolled activation of the clotting system in critically ill patients, with a potentially unfavorable role in their prognosis. In line with this hypothesis, we tested the possibility that the maintenance of normal AT levels by infusion of AT concentrate could have a beneficial effect on survival of critically ill patients irrespective of the causes leading to the requirement for hemodynamic and/or respiratory support.

The study was randomized and double blind, with the inclusion of a placebo control arm. Identification of the infused material by the attending physicians was prevented by the use of identical black bottles, syringes and infusion-sets. Patients were included in the study if they were 18 to 75 years old, were admitted to the intensive care unit (ICU) because of sepsis and/or post-operative complications requiring respiratory and/or hemodynamic support and had plasma AT activity < 70% of normal. Septic shock was defined as sepsis-related hypotension requiring vasoactive drugs for more than 24 hours, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ failure. The respiratory support consisted of assisted or controlled ventilation for more than 24 hours. The hemodynamic support consisted of the administration of inotropic (dopamine or dobutamine, >5 μg/kg/min) and/or vasoactive amines (epinephrine or norepinephrine).

Patients were excluded if they had suffered multiple trauma, had liver cirrhosis or acute liver failure, cancer in terminal phase, immunodeficiency, or leukemia, if they were pregnant, or were being submitted to heparin therapy for hemodialysis, hemofiltration or other indications. Patients receiving heparin prophylaxis were not excluded.

The AT concentrate and the placebo (albumin solution, 50 g/L) were supplied by the manufacturer (Immuno) in identical black bottles containing either 2,000 U of AT or 2 g of albumin in lyophilized form. A fixed dose of 4,000 units of AT or 4 g of albumin were injected as a bolus in 30 min, followed by 1 bottle every 12 hours for 5 days by a pump-driven syringe. There was no limitation to standard medical care in each ICU, but for the infusion of fresh frozen plasma, indicated for patients with active bleeding and/or with PT ratios > 2.0, or of platelet concentrates, which were administered at the dosage of 1 unit/10 kg body weight if the platelet count was < 50×10^9/L.

The simplified acute physiologic score (SAPS)[48] was recorded in each patient at admission; a modified multi-organ failure (MOF) score,[47,49] was recorded at admission and daily thereafter for 7 days. Baseline AT determinations for the enrolment of patients were carried out locally in each hospital. Thereafter, no local AT determinations were permitted. AT data reported were obtained after centralized measurement against an established calibrator (Immuno).

The main end-point of the study was survival at 30 days. The sample size was calculated to detect a 50% reduction of the expected mortality in the placebo group (60%) with an α error of 0.05 and a β error of 0.10. This mortality figure was anticipated based on the results of a previous study validating the SAPS score in consecutive patients referred to ICUs.[48] No separate randomization blocks were applied for patients with or without septic shock.

One hundred and twenty consecutive patients were enrolled (60 in each treatment arm) from January 1991 to November 1994 in three ICUs: 92 patients because of post-operative complications, 12 patients because of bronchopneumonia with septic shock, and 16 patients with a miscellany of disorders. One hundred patients had sepsis and 56 had septic shock at admission. The distribution of patients in the two arms was well balanced except for the number of patients with septic shock (33 in the AT arm versus 23 in the placebo arm, p = 0.08) and for the baseline

Table 4. Controlled studies of antithrombin replacement therapy in septic shock and critically ill patients.

Author (ref.)	No. of patients	Mortality rate Standard treatment	Mortality rate AT replacement	Odds Ratio	95% C.I.
Blauhut *et al.*, 1985[34]	51	12%	15%	1.29	0.18-42.9
GISACID, 1990[41]	41	23%	29%	1.33	0.24-∞
Harper *et al.*, 1991[45]	50	32%	32%	1.00	0.26-3.85
Albert *et al.*, 1992[46]	33	31%	25%	0.73	0.11-4.40
Fourrier *et al.*, 1993[43]*	32	50%	28%	0.40	0.06-2.19
Lamy *et al.*, 1996[44]*	42	41%	25%	0.48	0.10-2.16

*Double blind studies.

MOF score (AT arm: 5.6±2.5; placebo arm 4.8±2.3, p = 0.08). As a result, more patients in the AT group required hemodynamic support (53 in the AT arm versus 42 in the placebo arm, p = 0.04). Forty-nine patients in the AT group and 51 patients in the placebo group had sepsis. The infectious agents were identified in 93 patients by blood, urine and bronchoaspirate cultures and were similarly distributed in the two treatment arms (46 Gram-positive: *S. Aureus, S. Epidermidis*; 44 Gram-negative, *P. Aeruginosa, Serratia, Actinobacter, Enterobacter, K. Pneumoniae, E. Coli*; 34 fungi: *C. Albicans, Aspergillus*; *cytomegalovirus* 1). In patients with septic shock, hemodynamic parameters at entry were similarly abnormal in the two treatment arms.

Some degree of dyshomogeneity between the baseline characteristics of patients enrolled in the three centers participating in the study was observed, with statistically significant differences affecting SAPS (p=0.003), and AT levels (p=0.004), and probably resulted from poor standardization of AT measurements between the 3 laboratories.

Four patients received therapy for less than 24 hours: 1 patient, in the placebo group, was transferred to an another hospital after the bolus infusion and 3 patients (2 in the ATIII group and 1 in the placebo group), included in the intention to treat analysis, died on the day of enrolment. The mean time interval from admission to the ICUs and enrolment into the study (5.0±6.5 days) was not different for patients allocated to AT or placebo. Significant bleeding, requiring transfusion of red blood cell packs and platelet concentrates occurred in 6 patients in the placebo group and 5 patients in the AT group. No differences were observed between the treatment arms with respect to transfusion requirements with fresh frozen plasma, platelet and red blood cell packs. No side effects possibly related to AT treatment were observed.

Changes in plasma AT concentrations were not observed either in patients receiving placebo or in patients receiving AT after the initial rise observed following the first bolus injection (range 98-101%).

Survival curves were calculated for the 119 (intention to treat) and the 116 patients after the exclusion of the early deaths. By Kaplan-Meier analysis survival was not different in the two arms. At day 30, 30 patients in the AT arm (50%) and 27 patients in the placebo arm (46%) were alive. Because of the unbalanced randomization for baseline variables potentially affecting survival, we analyzed; by the Kaplan-Meier approach, the influence on survival of the requirement for hemodynamic support, the presence of septic shock and the MOF score at entry. The presence of septic shock (p<0.0001) and the requirement for hemodynamic support (p<0.0001) were negatively associated with survival; 30-day mortality was 75% in patients with septic shock and 32% in patients without shock. In addition, among patients with an unfavorable outcome, 75% of patients with septic shock died by day 5, whereas the same percentage of deaths was recorded by day 22 in patients without septic shock.

The significant influence on mortality rates of variables imperfectly balanced by the randomization process, led us to analyze the net effect of treatment on 30-day mortality after adjusting for the presence of covariates in a Cox regression model.[50] In addition to the presence of sepsis, septic shock and the requirement for hemodynamic support, the baseline MOF score and plasma AT activity, the time to treatment, age and center were included as covariates in the model (Table 5). At multivariate analysis, AT replacement had a net beneficial effect on 30-day survival (OR = 0.56, p<0.02). Of the covariates analyzed, the presence of septic shock (p=0.0002) and the baseline MOF score (p=0.02) were negatively associated with survival, while plasma AT activity levels (p=0.003) were positively and independently associated with survival, which also differed according to the center (p=0.006).

Because the two treatment arms were unbalanced for septic shock, the interaction of treatment with septic shock was tested in the model and resulted to be significantly associated with survival (p<0.0001). After stratification of patients for the presence or absence of septic shock, a net effect of AT treatment on mortality was observed only in the septic shock group (OR = 0.43, Table 5). In patients with septic shock, a significant effect of AT treatment on survival was also shown by Kaplan-Meier analysis (p = 0.04). Septic shock patients receiving treatment had a 34% (95% CI: 19%-49%) probability of being alive at day 30, with a corresponding probability of 13% (95% CI: 0%-26%) for septic shock patients receiving placebo and a resulting 30% reduction in 30-day mortality. The reduction in mortality produced by AT replacement was even more apparent when excluding early deaths from the analysis (p=0.016).

Table 5. Variables independently affecting survival of critically ill patients by Cox hazard regression analysis (ref. #11c).

Variables selected	*Odds Ratio*	*95% C.I.*
All patients		
Baseline AT % activity	0.97*	0.95-0.99
Center	1.61	1.15-2.24
Septic shock	3.97	1.77-6.25
Treatment	0.56	0.31-0.91
Patients with septic shock		
Baseline AT % activity	0.97*	0.95-0.99
Center	1.53	0.99-2.36
Treatment	0.43	0.23-0.83

The model includes as variables: treatment, MOF, SAPS, and AT levels at baseline, requirement for hemodynamic support, centers, time from admission to ICU until enrolment in the study, sepsis, septic shock, age.
**For unitary increase in % antithrombin activity.*

Contributions and Acknowledgments

DG and ADA wrote the manuscript; FB, GP, AR and LR participated in the design of the study which is the major issue of the present review and they are listed in alphabetical order. All the authors read and approved the final version of this manuscript.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

Manuscript received November 6, 1998; accepted January 26, 1999.

References

1. Parrillo JE, Parker MM, Natanson C, et al. Septic shock: advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. Ann Intern Med 1990; 113:227-42.
2. Manship L, McMillin RD, Brown JJ. The influence of sepsis and multisystem and organ failure on mortality in the surgical intensive care unit. Am Surg 1984; 50:94-101.
3. American College of Chest Physicians/Society of Critical Care Medicine Consensus Conference. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. Chest 1992; 101: 1644-55.
4. Tilney NL, Bailey GL, Morgan AP, et al. Sequential system failure after rupture of abdominal aortic aneurysms. Ann Surg 1973; 178:117-21.
5. Baue AE. Multiple, progressive or sequential systems failure: a syndrome of the 1970s. Arch Surg 1975; 110:779-86.
6. Fahey III TJ, Tracey KJ. Cytokines, tumor necrosis factor and other mediators of sepsis. In: Carlson RW, Geheb MA, eds. Principles & Practice of Medical Intensive Care. Philadelphia, WB Saunders Company; 1993. p. 311-22.
7. Cunnion RE, Schaer GL, Parker MM, Natanson C, Parrillo JE. The coronary circulation in human septic shock. Circulation 1986; 73:637-44.
8. Dhainaut JF, Huyghebaert MF, Monsallier JF, et al. Coronary hemodynamics and myocardial metabolism of lactate, free fatty acids, glucose, and ketones in patients with septic shock. Circulation 1987; 75:533-41.
9. Marder VJ, Feinstein DI, Francis CW, Colman RW. Consumptive thrombohemorrhagic disorders. In: Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. Hemostasis and thrombosis. Basic principles and clinical practice. Philadelphia, JB Lippincott Company, 1994. p. 1023-63.
10. Dixon AC, Parrillo JE. Cardiovascular dysfunction in septic shock. In: Carlson RW, Geheb MA, eds. Principles and Practice of Medical Intensive Care. Philadelphia: WB Saunders Co.; 1993. p. 323-40.
11. Lefer AM, Martin J. Origin of the myocardial depressant factor in shock. Am J Physiol 1970; 218:1423-9.
12. Parrillo JE, Burch C, Shelhamer JH, Parker MM, Natanson C, Schuette W. A circulating myocardial depressant substance in humans with septic shock. J Clin Invest 1985; 76:1539-53.
13. Hansen-Flaschen JH. Adult respiratory distress syndrome. In: Carlson RW, Geheb MA, eds. Principles and Practice of Medical Intensive Care. Philadelphia: WB Saunders Co.; 1993. p. 816-26.
14. Kaplan RL, Sahn SA, Petty TL. Incidence and outcome of the respiratory distress syndrome in gram-negative sepsis. Arch Intern Med 1979; 139:867-9.
15. Sibbald WJ, Darrah WC. Multiple organ system failure in sepsis. In: Carlson RW, Geheb MA, eds. Principles and Practice of Medical Intensive Care. Philadelphia: WB Saunders Co.; 1993. p. 340-52.
16. Solomon MA, Natanson C. Treatment of sepsis and septic shock. In: Carlson RW, Geheb MA, eds. Principles and Practice of Medical Intensive Care. Philadelphia: WB Saunders Co.; 1993. p. 365-80.
17. Salvo I, DeCian W, Musicco M, et al. The Italian SEPSIS study: preliminary results on incidence and evolution of SIRS, sepsis, severe sepsis and septic shock. Int Care Med 1995; 21:S244-9.
18. Ziegler EJ, McCutchan JA, Fierer J, et al. Treatment of gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia Coli*. N Engl J Med 1982; 307:1225-30.
19. Ziegler EJ, Fisher CJ, Sprung CL, et al. Treatment of Gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin: a randomized, double blind, placebo-controlled trial. N Engl J Med 1991; 324:429-36.
20. Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 1985; 229:869-71.
21. Tracey KJ, Fong Y, Hesse DG, et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. Nature 1987; 330:662-4.
22. Hinshaw LB, Tekamp-Olson P, Chang ACK, et al. Survival of primates in LD100 septic shock following therapy with antibody to tumor necrosis factor (TNFα). Circ Shock 1990; 30:279-88.
23. Coalson JJ, Benjamin B, Archer LT, et al. Prolonged shock in the baboon subjected to infusion of *E. Coli* endotoxin. Circ Shock 1978; 5:423-37.
24. Taylor FB Jr, Chang ACK, Peer GT, et al. DEGR-Factor Xa blocks disseminated intravascular coagulation initiated by *Escherichia Coli* without preventing shock or organ damage. Blood 1991; 78:364-8.
25. Warr TA, Rao VM, Rapaport SI. Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor: effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity. Blood 1990; 75: 1481-9.
26. Triantaphyllopoulos DC. Effects of human antithrombin III on mortality and blood coagulation induced in rabbits by endotoxin. Thromb Haemost 1984; 51: 232-5.
27. Taylor FB Jr, Emerson TE Jr, Jordan R, Chang A, Blick KE. Antithrombin III prevents the lethal effects of *Escherichia Coli* infusion in baboons. Circ Shock 1988; 26:227-35.
28. Taylor FB Jr., Chang A, Esmon CT, D'Angelo A, Viganò D'Angelo S, Blick KE. Protein C prevents the coagulopathic and lethal effects of *E. Coli* infusion in the baboon. J Clin Invest 1987; 79:918-25.
29. Creasey AA, Chang AC, Feigen L, Wun TC, Taylor FB Jr., Hinshaw LB. Tissue factor pathway inhibitor reduces mortality from *Escherichia Coli* septic shock. J Clin Invest 1993; 91:2850-6.
30. Buller HR, Ten Cate JW. Acquired antithrombin III deficiency: laboratory diagnosis, incidence, clinical implications and treatment with antithrombin III concentrate. Am J Med 1989; 87(suppl 3B):44s-8s.
31. Blauhut.B, Necek S, Vinazzer H, Bergmann H. Substitution therapy with an antithrombin III concentrate

in shock and DIC. Thromb Res 1982; 27:271-8.
32. Hesselvik JF, Malm J, Dahlbäck B, Blomback M. Protein C, protein S and C4b-binding protein in severe infection and septic shock. Thromb Haemost 1991; 62:126-9.
33. Fourrier F, Chopin C, Goudemand J, et al. Septic shock, multi organ failure and disseminated intravascular coagulation. Compared pattern of ATIII, protein C, protein S deficiencies. Chest 1992; 101:816-23.
34. Blauhut B, Kramar H, Vinazzer H, Bergmann H. Substitution of antithrombin III in shock and DIC: a randomized study. Thromb Res 1984; 39:81-9.
35. Wilson RF, Mammen EF, Robson MC, Heggers JP, Soullier G, DePoli PA. Antithrombin, prekallikrein and fibronectin levels in surgical patients. Arch Surg 1986; 121:635-43.
36. Sandset PM, Roise O, Aasen AO, Abildgaard U. Extrinsic pathway inhibitor in post operative/ post traumatic septicemia: increased levels in fatal cases. Haemostasis 1989; 19:189-95.
37. Hesselvik JF, Blomback M, Brodin B, Maller R. Coagulation, fibrinolysis and kallikrein systems in sepsis: relation to outcome. Crit Care Med 1989; 17:724-33.
38. Lammle B, Tran TM, Ritz R, Duckert F. Plasma prekallikrein, factor XII, ATIII, C1-inhibitor and α2 macroglobulin in critically ill patients with suspected disseminated intravascular coagulation (DIC). Am J Clin Pathol 1984; 82:396-404.
39. Maki M, Terao T, Ikenoue T, et al. Clinical evaluation of antithrombin concentrate (BI 6.013) for disseminated intravascular coagulation in obstetrics. Gynecol Obstet Invest 1987; 23:230-40.
40. Vinazzer H. Therapeutic use of antithrombin III in shock and disseminated intravascular coagulation. Semin Thromb Hemost 1989; 15:347-52.
41. Palareti G, Legnani C, Ludovici S, et al. Studio preliminare per la valutazione di fattibilità di uno studio clinico policentrico per la valutazione dell'efficacia terapeutica di un concentrato di antitrombina III (Immuno) nella sindrome da coagulazione intravascolare. Haematologica 1990; 75:21-8.
42. Lechner K, Kyrle PA. Antithrombin III concentrates - Are they clinically useful? Thromb Haemost 1995; 73: 340-8.
43. Fourrier F, Chopin C, Huart JJ, Runge I, Caron C, Goudemand J. Double-blind, placebo-controlled trial of antithrombin III concentrates in septic shock with disseminated intravascular coagulation. Chest 1993; 104:882-8.
44. Lamy M, Eisele B, KeineckeH-O, Delvos U, Thijs LG. Antithrombin III in patients with severe sepsis. A randomised, placebo-controlled trial of antithrombin III concentrates in septic shock with disseminated intravascular coagulation [abstract]. 9th European Congress on Intensive Care Medicine, Glasgow, 1996. p. 385-90.
45. Harper PL, Williamson L, Park G, Smith JK, Carrell RW. A pilot study of antithrombin replacement in intensive care management: the effects on mortality, coagulation and renal function. Transfusion Med 1991; 1:121-8.
46. Albert J, Blomqvist H, Gardlund B, Jakobsson J, Svensson J, Blomback M. Effect of antithrombin concentrate on haemostatic variables in critically ill patients. Acta Anaesthesiol Scand 1992; 36:745-52.
47. Baudo F, Caimi TM, de Cataldo F, et al. Antithrombin III (AT III) replacement therapy in patients with sepsis and/or postsurgical complications: a controlled double blind, randomized multicenter study. Int Care Med 1998; 24:336-42.
48. La Gall JR, Loirat P, Alperovith A, et al. A simplified acute physiology score for ICU patients. Crit Care Med 1984; 12:975-7.
49. Goris RJA, Boekhorst TP, Nuytinck JKS, Gimbrere JSF. Multiple organ failure. Arch Surg 1985; 120:1109-15.
50. Christensen E. Multivariate survival analysis using Cox's regression model. Hepatology 1987; 7:1346-58.
51. Mesters RM, Mannucci PM, Coppola R, Keller T, Ostermann H, Kienast J. Factor VIIa and antithrombin III activity during severe sepsis and septic shock in neutropenic patients. Blood 1996; 88:881-6.
52. Smtih OP, White B, Vaughan D, et al. Use of protein-C concentrate, heparin, and haemodiafiltration in meningococcus-induced purpura fulminans. Lancet 1997; 350:1590-3.
53. Fijnvandraat K, Derkx B, Peters M, et al. Coagulation activation and tissue necrosis in meningococcal septic shock: severely reduced protein C levels predict a high mortality. Thromb Haemost 1995; 73:15-20.
54. Esmon CT, Schwarz HP. An update on clinical and basic aspects of the protein C anticoagulant pathway. Trends Cardiovasc Med 1995; 5:141-8.

Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin

Mark L. Kahn,[1] Mayumi Nakanishi-Matsui,[1] Michael J. Shapiro,[1] Hiroaki Ishihara,[2] and Shaun R. Coughlin[1,3]

[1]Cardiovascular Research Institute and Daiichi Research Center, University of California–San Francisco, San Francisco, California 94143-0130, USA
[2]New Product Research Laboratories II, Daiichi Pharmaceutical Company, Ltd., Tokyo 134, Japan
[3]Departments of Medicine and Cellular and Molecular Pharmacology, University of California–San Francisco, San Francisco, California 94143-0130, USA

Address correspondence to: Shaun R. Coughlin, University of California–San Francisco, HSE-1300, Box 0130, 505 Parnassus Avenue, San Francisco, California 94143-0130, USA. Phone: (415) 476-6174; Fax: (415) 476-8173; E-mail: shaun_coughlin@quickmail.ucsf.edu

Mark L. Kahn's present address is: University of Pennsylvania, 809 Stellar Chance Laboratories, Philadelphia, Pennsylvania 19104, USA.

Received for publication December 14, 1998, and accepted in revised form January 28, 1999.

Because of the role of thrombin and platelets in myocardial infarction and other pathological processes, identifying and blocking the receptors by which thrombin activates platelets has been an important goal. Three protease-activated receptors (PARs) for thrombin — PAR1, PAR3, and PAR4 — are now known. PAR1 functions in human platelets, and the recent observation that a PAR4-activating peptide activates human platelets suggests that PAR4 also acts in these cells. Whether PAR1 and PAR4 account for activation of human platelets by thrombin, or whether PAR3 or still other receptors contribute, is unknown. We have examined the roles of PAR1, PAR3, and PAR4 in platelets. PAR1 and PAR4 mRNA and protein were detected in human platelets. Activation of either receptor was sufficient to trigger platelet secretion and aggregation. Inhibition of PAR1 alone by antagonist, blocking antibody, or desensitization blocked platelet activation by 1 nM thrombin but only modestly attenuated platelet activation by 30 nM thrombin. Inhibition of PAR4 alone using a blocking antibody had little effect at either thrombin concentration. Strikingly, simultaneous inhibition of both PAR1 and PAR4 virtually ablated platelet secretion and aggregation, even at 30 nM thrombin. These observations suggest that PAR1 and PAR4 account for most, if not all, thrombin signaling in platelets and that antagonists that block these receptors might be useful antithrombotic agents.

J. Clin. Invest. **103**:879–887 (1999).

Introduction

Platelet activation is critical for normal hemostasis, and platelet-dependent arterial thrombosis underlies most myocardial infarctions. Thrombin is the most potent activator of platelets (1, 2). Characterization of the receptors that mediate thrombin's actions on platelets is therefore necessary for understanding hemostasis and thrombosis. Moreover, such receptors are potential targets for novel antiplatelet therapies.

Thrombin signaling is mediated at least in part by a family of G protein–coupled protease-activated receptors (PARs), for which PAR1 is the prototype (3, 4). PAR1 is activated when thrombin cleaves its NH_2-terminal exodomain to unmask a new receptor NH_2-terminus (3). This new NH_2-terminus then serves as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect transmembrane signaling (3, 5, 6). The synthetic peptide SFLLRN, which mimics the first six amino acids of the new NH_2-terminus unmasked by receptor cleavage, functions as a PAR1 agonist and activates the receptor independent of proteolysis (3, 7, 8). Such peptides have been used as pharmacological probes of PAR function in various cell types.

Our understanding of the role of PARs in platelet activation is evolving rapidly. PAR1 mRNA and protein were detected in human platelets (3, 9–11), SFLLRN activated human platelets (3, 7, 8), and PAR1-blocking antibodies inhibited human platelet activation by low, but not high, concentrations of thrombin (9, 10). These data suggested a role for PAR1 in activation of human platelets by thrombin but left open the possibility that other receptors might contribute.

Curiously, PAR1 appears to play no role in mouse platelets. PAR1-activating peptides did not activate rodent platelets (12–14), and platelets from PAR1-deficient mice responded like wild-type platelets to thrombin (14). This observation prompted a search for additional thrombin receptors and led to the identification of PAR3 (15). PAR3 is activated by thrombin and is expressed in mouse platelets. PAR3-blocking antibodies inhibited mouse platelet activation by low, but not high, concentrations of thrombin (16), and knockout of PAR3 abolished mouse platelet responses to low, but not high, concentrations of thrombin (17). These results established that PAR3 is necessary for normal thrombin signaling in mouse platelets but also pointed to the existence of another mouse platelet thrombin receptor. Such a receptor, PAR4, was recently identified (17, 18). PAR4 appears to function in both mouse and human platelets (17). Thus, available data suggest a testable working model in which PAR3 and PAR4 mediate thrombin activation

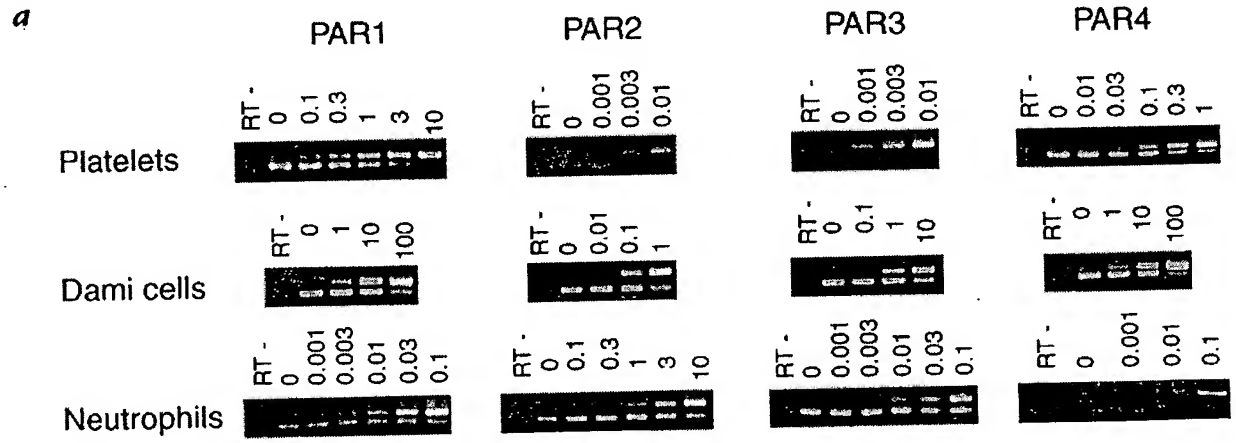
of mouse platelets and PAR1 and PAR4 mediate activation of human platelets. The role of PAR3, if any, in human platelets has not been determined. More broadly, the relative roles of PAR1, PAR3, and PAR4, and whether still other receptors also contribute to platelet activation by thrombin, are unknown.

To determine the roles of PAR1, PAR3, and PAR4 in activation of human platelets by thrombin, we examined expression of receptor mRNA and protein in platelets and probed receptor function with specific peptide agonists. We also examined the effect of receptor desensitization, receptor-blocking antibodies, and a PAR1 antagonist, used alone and in combination, on platelet activation. Our results suggest that PAR1 and PAR4 together account for most, if not all, thrombin signaling in human platelets. PAR3, while important for thrombin signaling in mouse platelets, appears to have little or no role in human platelets. These results are potentially important for the development of antiplatelet therapies.

Methods

Measurement of PAR mRNA levels by competitive reverse transcription-PCR. Dami cells (19) were grown in suspension in RPMI with 10% FBS. Platelet preparations (17) contained <0.1% leukocytes as assessed by light microscopic analysis. A discontinuous Percoll gradient was used to separate monocytes and lymphocytes from neutrophils, according to the manufacturer's instructions (Pharmacia Biotech Inc., Piscataway, New Jersey, USA). The monocyte/lymphocyte preparations contained <0.1% neutrophils, and the neutrophil preparations contained <0.1% monocytes or lymphocytes. Total RNA was prepared from all cells using Trizol Reagent (GIBCO BRL, Grand Island, New York, USA), treated with DNase (Boehringer Mannheim, Indianapolis, Indiana, USA), and quantified by OD 260.

Competitor RNA templates for each receptor were created by mutating the respective cDNA to ablate an endogenous restriction endonuclease site (see below), and competitor RNAs were generated by *in vitro* transcription. Reverse transcription (RT) reactions were performed using 200 ng of total cellular RNA mixed with varying amounts of competitor RNA in a 10-µl reaction volume using a commercial kit (GIBCO BRL) and receptor specific primers (see below). RT product (2 µl) was amplified by PCR in a 50-µl volume containing a final concentration of 2 µM primers (see below) and 5 U *Taq* polymerase (GIBCO BRL). Reaction conditions were 94°C for 4 min, 72°C for 1 min with addition of *Taq*, then 94°C for 45 s, 55°C for 1 min, 72°C for 1 min for 30–36 cycles (see below), and then 72°C for 8 min. Cycle numbers and concentration ranges for competitor RNAs were chosen for each sample in preliminary experiments. The number of cycles chosen for measurement of PAR1, PAR2, PAR3, and PAR4 mRNA levels, respectively, in RNA from the various sources follows.



b

	PAR1	PAR2	PAR3	PAR4
	(attomoles/200 ng total RNA)			
Platelets (1)	1	0.001	< 0.001	0.3
(2)	0.3 - 1	< 0.001	< 0.001	0.1
Dami cells	10	0.1	1 - 10	10
Neutrophils	0.01	3	0.03 - 0.1	< 0.01
Monocytes + Lymphocytes	1	0.3	0.03 - 0.1	0.3

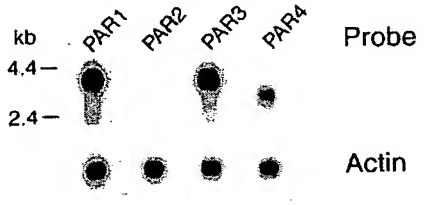


Figure 1
Expression of mRNAs encoding PAR1, PAR2, PAR3, and PAR4 in platelets, Dami cells, and neutrophils. (*a*) Competitive RT-PCR of total RNA from platelets, Dami cells, and neutrophils. Total cellular RNA (200 ng) mixed with the indicated quantity of competitor RNA (measured in attomoles) was reverse-transcribed and amplified. Products were digested with a restriction endonuclease to distinguish the products of competitor RNA (*uncleavable upper band*) vs. native cellular mRNA (*lower band*). RT indicates mock RT-PCR of total cellular RNA and the highest amount of competitor RNA with no reverse transcriptase added. Each sample was analyzed at least twice. Note that the single band seen in the platelet PAR3 RT-PCR is due to amplification of competitor RNA. (*b*) Quantitation of PAR mRNAs in platelet, Dami cell, neutrophil, and monocyte/lymphocyte preparations. Results indicate the range of values obtained from at least two experiments like that shown in *a*. 1 amol/200 ng corresponds to an mRNA relative abundance of roughly 1:3,000. The expression of PAR mRNA in the platelets of two unrelated individuals is shown. (*c*) Northern blot analysis of PAR gene expression in Dami cells. Blots were hybridized separately with coding region probes for PAR1, PAR2, PAR3, or PAR4, as well as with probe for β-actin mRNA as a control for lane loading. Note concordance with PCR data in *b*. *PAR*, protease-activated receptor; *RT*, reverse transcription.

Platelets: 31, 36, 36, 36; neutrophils: 36, 27, 31, 36; monocytes/lymphocytes: 31, 31, 33, 36; and Dami cells: 30, 32, 32, 33.

Primers used for RT and PCR of each receptor and the restriction endonuclease used to digest each PCR product. Nucleotide numbering is such that 1 equals the A of the start ATG.

PAR1, GenBank accession no. M62424: Primer for RT: TAG ACG TAC CTC TGG CAC TC (1148-1129). Sense-strand primer for PCR: CAG TTT GGG TCT GAA TTG TGT CG. Antisense primer for PCR: TGC ACG AGC TTA TGC TGC TGA C. Resulting PCR product: 505-1096. Mutated site: *Age*I at position 596.

PAR2, GenBank accession no. U34038: Primer for RT: CTG CTC AGG CAA AAC ATC (699-682). Sense-strand primer for PCR: TGG ATG AGT TTT CTG CAT CTG TCC. Antisense primer for PCR: CGT GAT GTT CAG GGC AGG AAT G. Resulting PCR product: 182-672. Mutated site: *Sfi*I at position 342.

PAR3 GenBank accession no. U92972: Primer for RT: TGA TGT CTG GCT GAA CAA G (727-709). Sense-strand primer for PCR: TCC CCT TTT CTG CCT TGG AAG. Antisense primer for PCR: AAA CTG TTG CCC ACA CCA GTC CAC. Resulting PCR product: 152-664. Mutated site: *Nco*I at position 251.

PAR4, GenBank accession no. AF080214: Primer for RT: TGA GTA GCT GGG ATT ACA G (1519-1501). Sense-strand primer for PCR: AAC CTC TAT GGT GCC TAC GTG C. Antisense primer for PCR: CCA AGC CCA GCT AAT TTT TG. Resulting PCR product: 949-1490. Mutated site: *Bam*HI at position 1005.

After PCR amplification, 10 μl of reaction product was digested overnight with the appropriate restriction endonuclease and analyzed by 1.5% agarose gel electrophoresis. The products of reactions that included only native mRNA were completely cleaved by the appropriate restriction endonuclease, while the products of reactions that included only competitor RNA remained undigested (Fig. 1 and data not shown). By adding varying amounts of competitor RNA to total cellular RNA before RT-PCR and determining the competitor RNA concentration at which the intensity of the competitor RNA-derived product (uncleaved band) matched that of the endogenous mRNA-derived product (cleaved band), the quantity of each PAR mRNA in the original sample was estimated.

Northern blot analysis. Poly(A)$^+$ RNA (2 μg) derived from Dami cells was electrophoresed, transferred to nitrocellulose membranes, and hybridized under high-stringency conditions. PAR1 mRNA was detected with a 400-bp *Pst*I/*Pvu*II cDNA probe; PAR2 mRNA was detected with a 260-bp *Sfi*I/*Bst*EII cDNA probe; PAR3 mRNA was detected with a 610-bp *Kpn*I/*Nsi*I cDNA probe; PAR4 mRNA was detected using a 450-bp *Sac*I/*Pst*I cDNA.

Generation and characterization of PAR polyclonal antibodies. The synthetic peptides GGDDSTPSILPAPRGYPGQVC (PAR4 amino acids 34–55), AKPTLPIKTFRGAPPNSFEEFPFSALEGC (PAR3 amino acids 31–58 plus carboxyl glycine-cysteine) and NATLDPRSFLLRNPNDKYEPFWEDEEGC (PAR1 amino acids 35–61 plus carboxyl glycine-cysteine) were conjugated to keyhole limpet hemocyanin and used to generate polyclonal antisera in rabbits. IgG was purified by protein-A affinity chromatography to generate the PAR4, PAR3, and PAR1 IgG preparations used in this study. Binding of these IgGs and PAR4 preimmune IgG to each receptor was tested on COS cells transiently expressing FLAG epitope-tagged receptors using an enzyme-linked immunosorbent assay (ELISA) (16, 20). cDNA for an epitope-tagged human PAR4 analogous to FLAG epitope-tagged PAR1 was constructed as described previously (15, 20) such that the FLAG epitope was fused to amino acid 22 in PAR4 to yield the following sequence: ... DYKDDDDVE/TPSVYD ... (where the slash indicates the junction with PAR4 sequence).

Flow cytometry. Washed platelets (17) and Dami cells were fixed with paraformaldehyde, incubated with PAR1 or PAR3 IgG at 10 μg/ml or PAR4 IgG at 100 μg/ml, washed, incubated with FITC-conjugated goat anti-rabbit IgG, washed, and then analyzed in a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, California, USA). Some fixed platelet samples were exposed to 30 nM thrombin at 37°C before incubation with primary antibody.

Functional studies in Xenopus oocytes. FLAG epitope-tagged PAR4 cDNA was subcloned into pFROG (3) to permit *in vitro* transcription of PAR4 cRNA. Signaling studies were performed in *Xenopus* oocytes microinjected with 2.0 ng of PAR4 cRNA or 25 ng of PAR1 cRNA per oocyte (3, 21).

Platelet aggregation and secretion. Aggregation and secretion were measured using washed human platelets (17). For desensitization studies, platelets resuspended from the first platelet pellet were incubated with SFLLRN (100 μM) or GYPGKF (500 μM) in the presence of prostaglandin E_1 (PGE_1) at room temperature for 30 min without stirring then washed by centrifugation (17).

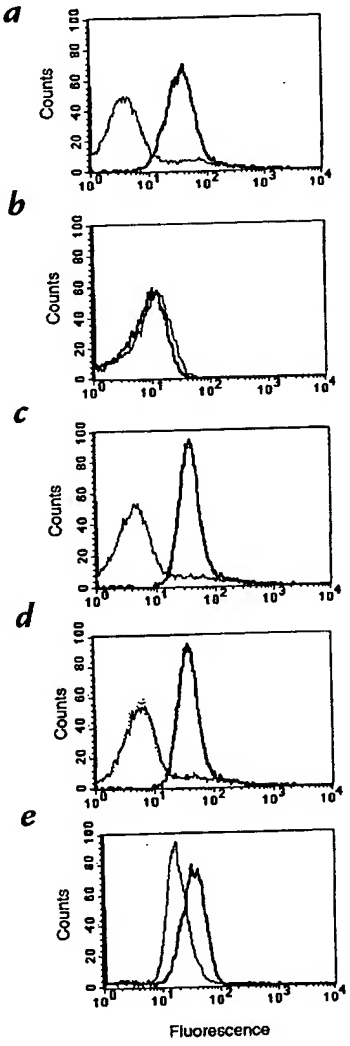


Figure 2
Flow cytometric analysis of platelets for surface expression of PAR1, PAR3, and PAR4. Fixed platelets were incubated with preimmune IgG (*narrow lines*) or PAR1 IgG (*a*), PAR3 IgG (*b*), or PAR4 IgG (*c*) (*wide lines*) and then analyzed as described in Methods. (*d*) Platelets were incubated with PAR4 IgG in the absence (*wide line*) or presence (*thin line*) of the peptide antigen (1 μM) used to generate the PAR4 antiserum, or after treatment with 20 nM thrombin for 10 min at 37°C (*dotted line*). Each curve represents an analysis of 10,000 events. This experiment was repeated twice with separate donors with equivalent results. (*e*) Flow cytometric analysis of Dami cells as a positive control for detection of PAR3. Fixed Dami cells were incubated with preimmune IgG (*narrow line*) or PAR3 IgG (*wide line*) and then analyzed as above. Dami cells were also positive for PAR1 and PAR4 (not shown).

For functional studies with PAR1 or PAR4 antibody, washed platelets were incubated with antibody or preimmune IgG for 60 min before measurement of secretion and aggregation. PAR1 antagonist was added to stirring platelets 1–2 min before the addition of thrombin or other agonists.

Measurement of receptor cleavage by thrombin. Rat-1 fibroblasts were stably transfected with FLAG epitope-tagged PAR1 and PAR4 expression vectors (22), and cleavage of surface receptors was followed as described previously (20).

Measurement of PAR1 and PAR4 signaling. A *Par1*$^{-/-}$ mouse lung fibroblast cell line that showed no thrombin signaling (14, 23) was used to generate stable cell lines expressing FLAG epitope-tagged PAR1 and PAR4. Increases in cytoplasmic calcium in response to thrombin were measured using the calcium-sensitive dye Fura-2 as described previously (14).

Results

Expression of PAR mRNAs in platelets and other blood cells. To validate an RT-PCR assay for PAR mRNAs, Dami cells, a human cell line that expresses some megakaryocyte markers (19), were analyzed. Competitive RT-PCR of Dami cell RNA (Fig. 1, *a* and *b*) revealed the presence of PAR1, PAR3, and PAR4 mRNA; PAR2 mRNA was also detected but at only 1% the level of PAR1 mRNA. Northern analysis was positive for PAR1, PAR3, and PAR4 but not PAR2 (Fig. 1*c*). At the protein level, PAR1, PAR3, and PAR4 were detected on the surface of Dami cells by flow cytometry (Fig. 2, and data not shown). Thus, results from RT-PCR were generally concordant with Northern and protein analysis.

Competitive RT-PCR of platelet RNA revealed PAR1 mRNA to be present at approximately 1 amol/200 ng total RNA. Assuming mRNA is 1% of total platelet RNA and an average mRNA size of 2 kb, PAR1 mRNA represents 1 in 3,000 platelet mRNAs. PAR4 mRNA was also detected at 10%–30% of PAR1 mRNA levels. By contrast, PAR3 mRNA was undetectable. PAR3 competitor RNA added to platelet RNA was detected at 0.001 amol/200 ng total RNA, suggesting that PAR3 mRNA was at least 1,000-fold less abundant than PAR1 mRNA in these samples. PAR2 mRNA was not detected in platelet RNA from one individual (no. 2), and only 0.001 amol/200ng was detected in the other (no. 1). The latter measurement may be due to trace contamination of the platelet preparation by neutrophils (see below), consistent with the observation that the specific PAR2 agonist peptide SLIGKV is unable to activate human platelets (data not shown).

The pattern of PAR mRNA expression in neutrophils and mononuclear cells was distinct from that seen in platelets, suggesting that contamination of platelet preparations by leukocytes did not significantly influence the PAR expression pattern detected in platelets. In particular, while virtually absent from platelets, substantial PAR2 mRNA was detected in both neutrophils and mononuclear cells. The relatively high PAR2 mRNA level in neutrophils is consistent with previous studies demonstrating neutrophil responses to PAR2-activating peptide (24). In contrast to platelets, PAR3 mRNA was consistently detected at low levels in mononuclear cells. PAR4 mRNA was also found in mononuclear cell preparations but not in neutrophils. These results demonstrate the presence of mRNA encoding PAR1 and PAR4, but not PAR2 or PAR3, in human platelets.

Expression of PAR proteins on the surface of human platelets. IgG was purified from rabbit antisera raised to peptides representing the NH_2-terminal exodomains of PAR1, PAR3, or PAR4. To assess ability to recognize native PARs and cross-reactivity, antibody binding to the surface of receptor-expressing COS cells was measured. Each IgG preparation bound to the surface of cells expressing the appropriate receptor without significant cross-reactivity (data not shown).

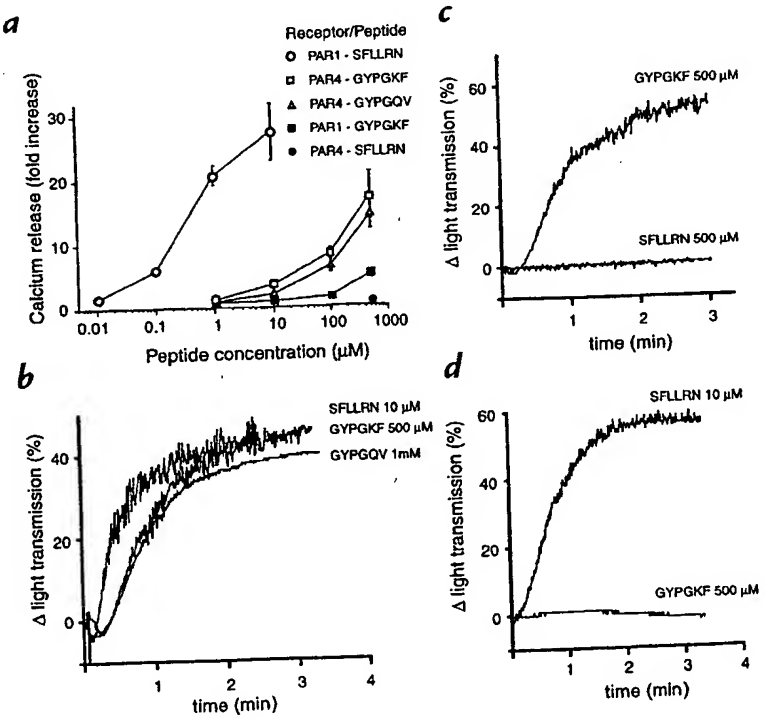


Figure 3
Effects of PAR1- and PAR4-activating peptides. (*a*) Specificity and potency. Peptide-triggered ^{45}Ca release was measured in *Xenopus* oocytes expressing human PAR1 and human PAR4 tagged at their NH_2-termini with a FLAG epitope. Data are mean ± SEM ($n = 3$) and are expressed as fold increase over baseline for each receptor. Surface expression of PAR1 measured with anti-FLAG monoclonal antibody was 1.3 times that of PAR4. This experiment was replicated twice. (*b–d*) Activation of human platelets with the PAR1-activating peptide SFLLRN and the PAR4-activating peptides GYPGKF and GYPGQV. (*b*) Platelets were exposed to either SFLLRN (10 μM) or GYPGKF (500 μM) or GYPGQV (1 mM) at time 0, and aggregation was measured as change in light transmission. (*c*) SFLLRN-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) at time 0, and aggregation was measured as change in light transmission. (*d*) GYPGKF-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) at time 0, and aggregation was measured as change in light transmission. The experiments in *b*, *c*, and *d* were repeated three times.

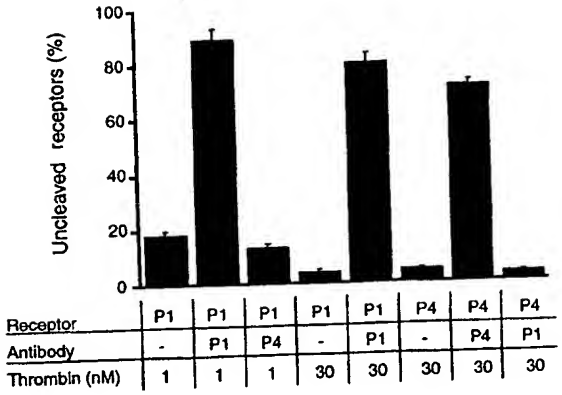


Figure 4
Inhibition of thrombin cleavage of receptor NH_2-terminus by anti-PAR1 and anti-PAR4 antibodies. Rat-1 cells expressing PAR1 and PAR4 bearing the FLAG epitope at their extreme NH_2-termini were fixed and then incubated with PAR1 IgG (*P1*; 100 μg/ml), PAR4 IgG (*P4*; 1 mg/ml), or buffer alone for 60 min before exposure to either 1 or 30 nM thrombin for 10 min at 37°C. Receptor cleavage was measured as loss of binding sites for M1 monoclonal antibody to the FLAG epitope, which was NH_2-terminal to the thrombin cleavage site in both receptors, so as to be lost from the cells upon receptor cleavage. Data (mean ± SEM; $n = 3$) are expressed as percent of control cells exposed to buffer alone. This experiment was repeated twice.

These IgG preparations were then used for flow cytometric analysis of human platelets (Fig. 2). Significant surface binding was detected with PAR1 IgG vs. preimmune IgG (Fig. 2*a*), consistent with previous studies (9, 11, 25, 26). A similar increase in platelet surface binding was detected with PAR4 IgG vs. PAR4 preimmune IgG (Fig. 2*c*). Preincubation of PAR4 IgG with the peptide antigen to which it was raised abolished this increase (Fig. 2*d*). Moreover, the epitope to which the PAR4 antiserum was raised spans PAR4's thrombin cleavage site, and treatment of platelets with thrombin indeed abolished PAR4 IgG binding (Fig. 2*d*). These data strongly suggest that PAR1 and PAR4 are expressed on the surface of human platelets.

PAR3 immune IgG showed no specific binding to human platelets (Fig. 2*b*). To determine whether this antibody preparation could detect PAR3 expressed at "natural" levels, this experiment was repeated with Dami cells (Fig. 2*e*, and data not shown), which had been shown by Northern blot to express PAR3 mRNA (Fig. 1). A significant increase in antibody binding was seen with PAR3 antibody vs. nonimmune IgG, consistent with RT-PCR and Northern blot analysis (Fig. 1). This suggests that the absence of detectable PAR3 protein on the surface of human platelets is not due to insensitivity of the assay. Taken together, these data confirm the presence of PAR1 and PAR4, but not PAR3, on the surface of human platelets.

Activation of human platelets by PAR1- and PAR4-activating peptides. Synthetic peptides that mimic the tethered ligands of PAR1 and PAR2 function as agonists for their respective receptors (3, 7, 8) and have been used as pharmacological tools to probe the function of these receptors in various cell types. Unfortunately, the cognate peptide for PAR3 appears to be insufficiently avid to function as a free ligand (15). We and others (17, 18) recently showed that a peptide mimicking the tethered ligand for PAR4 can function as an agonist for that receptor, albeit at a concentration higher than that seen with the PAR1 and PAR2 peptides and their cognate receptors. To determine the specificity of the PAR1 and PAR4 tethered ligand peptides, we first assessed their ability to trigger calcium mobilization in *Xenopus* oocytes heterologously expressing PAR1 and PAR4, our most sensitive assay system (Fig. 3*a*). No responses were detected in oocytes expressing neither receptor (not shown). Both the human PAR4 peptide GYPGQV and the mouse PAR4 peptide GYPGKF activated oocytes expressing human PAR4, but with an EC_{50} roughly two orders of magnitude higher than that of SFLLRN for PAR1 activation (Fig. 3*a* and ref. 17). SFLLRN showed no activity at PAR4. At 500 μM, the PAR4 peptide GYPGKF did show minimal activity at PAR1. However, because PAR1 is overexpressed such that the sensitivity for detection of PAR1 activation in the oocyte assay is 10- to 100-fold greater than in platelets, it is likely that PAR1 activation at 500 μM GYPGKF is unimportant in the platelet studies described below.

The PAR1 peptide SFLLRN and the PAR4 peptides GYPGKF and GYPGQV all activated human platelets (Fig. 3*b*). The PAR4 peptides were considerably less potent than the PAR1 peptide for activating human platelets; GYPGKF was slightly more potent than GYPGQV (Fig. 3*b*, and data not shown). The potencies of these peptides for platelet activation thus correlated with their relative potencies for activation of their respective receptors in the oocyte system (Fig. 3).

Incubation of PGE_1-treated platelets with SFLLRN rendered them refractory to subsequent stimulation by SFLLRN but did not affect responsiveness to GYPGKF (Fig. 3*c*). Conversely, incubation with GYPGKF rendered platelets refractory to subsequent stimulation by GYPGKF but did not affect responsiveness to SFLLRN (Fig. 3*d*). These results suggest that activation of either PAR1 or PAR4 with their cognate peptide agonists is sufficient to activate human platelets. Taken together, the results presented above show that PAR1 and PAR4 function in human platelets.

PAR1 and PAR4 antibodies inhibit thrombin cleavage of their respective receptors. To determine the necessary roles of PAR1 and PAR4 in platelet activation by thrombin, we developed blocking antibodies. The previously described PAR1 antibody raised against PAR1's hirudin-like domain (9) is predicted to inhibit thrombin cleavage of PAR1's NH_2-terminal exodomain by disrupting binding to thrombin's anion-binding exosite. Because no analogous hirudin-like domain was apparent in the sequence of PAR4's NH_2-terminal exodomain, antiserum was raised to a peptide that represented sequence spanning PAR4's thrombin cleavage site. This antiserum specifically recognized PAR4 (Fig. 2, and data not shown). To test the ability of the PAR1 and PAR4 antibodies to block cleavage of PAR1 and PAR4, Rat-1 fibroblasts expressing FLAG epitope–tagged PAR1 and PAR4 were preincubated with antibody. Receptor cleavage was then measured as loss of FLAG epitope from the cell surface upon expo-

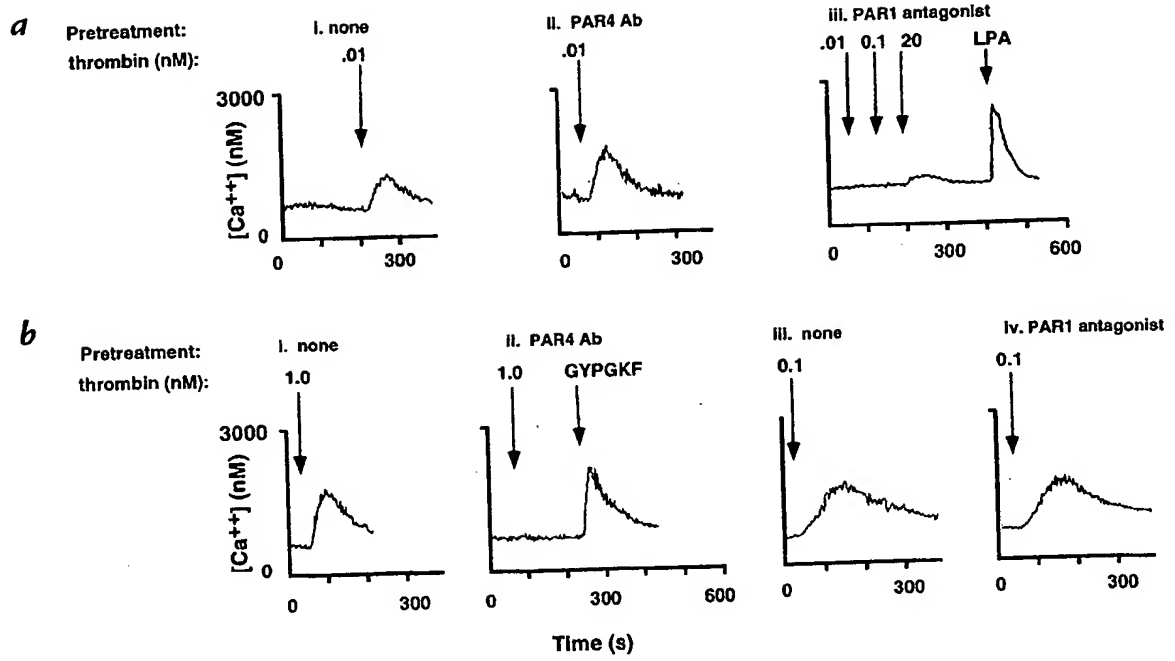


Figure 5
Inhibition of PAR1 and PAR4 signaling by PAR1- and PAR4-blocking antibodies and PAR1 antagonist. Fibroblast cell lines in which thrombin signaling was mediated solely by PAR1 (*a*) or by PAR4 (*b*) were incubated with buffer alone (*none*), PAR4 IgG (*PAR4 Ab*; 1 mg/ml), or the PAR1 antagonist BMS200261 (100 μM) for 30 min at 37°C. Cells were then exposed to thrombin (0.01, 0.1, 1.0, or 20 nM as indicated), GYPGKF (500 μM), or lysophosphatidic acid (*LPA*; 5 μM). Receptor-triggered increases in cytoplasmic calcium were measured fluorometrically using the calcium sensitive dye Fura-2. This experiment was repeated three times with similar results. *Ab*, antibody.

sure to thrombin (20) (Fig. 4). PAR1 cleavage was markedly inhibited by PAR1 antibody but not by PAR4 antibody. Conversely, PAR4 cleavage was markedly inhibited by PAR4 antibody, but not by PAR1 antibody. These data predicted that the PAR1 and PAR4 antibodies should selectively attenuate thrombin signaling via PAR1 and PAR4, respectively.

Inhibition of thrombin signaling by PAR1 and PAR4 antibodies, and by a PAR1 antagonist. A fibroblast cell line derived from PAR1-deficient mice (23) was used to generate lines stably expressing human PAR1 and PAR4. Because no thrombin responses were detectable in untransfected PAR1-deficient fibroblasts, signaling in the transfected cells could be attributed to the transfected receptor. In the PAR1-expressing cell line, increases in cytoplasmic calcium were reliably elicited by thrombin at concentrations as low as 10 pM (Fig. 5*a*). PAR4 IgG had no inhibitory effect, even on these threshold responses (Fig. 5). As demonstrated previously (9), PAR1 IgG markedly attenuated such signaling, and nonimmune antibody was without effect (data not shown).

The PAR1 antagonist BMS200261 (27) attenuated PAR1 signaling even at high thrombin concentrations (Fig. 5). Responsiveness to lysophosphatidic acid was unaffected by the antagonist, as was PAR4 signaling (Fig. 5, and data not shown), suggesting that the inhibitory effect of BMS200261 was specific.

In the PAR4-expressing cell line, increases in cytoplasmic calcium were reliably triggered at 1 nM thrombin (Fig. 5*b*). PAR4 IgG blocked such responses but had no effect on responses to GYPGKF, consistent with the antibody's acting by preventing receptor cleavage by thrombin. PAR4 preimmune IgG, PAR1 IgG, and PAR1 antagonist (100 μM) failed to inhibit PAR4 signaling even at threshold thrombin concentrations (Fig. 5*b*, and data not shown). Taken together, these results established specific tools for blocking PAR1 or PAR4. PAR1 and PAR4 could each be blocked with a specific IgG. PAR1 could also be specifically blocked with BMS200261 or by homologous desensitization with SFLLRN. This presented an opportunity to assess the roles of PAR1 and PAR4 in platelet activation by thrombin.

Inhibition of thrombin-induced platelet aggregation by blocking PAR1 and PAR4 signaling. The contribution of PAR1 and PAR4 signaling to thrombin activation of human platelets was tested using the strategies outlined above. By itself, PAR4 IgG had no effect on platelet aggregation, even at low (1 nM) thrombin (Fig. 6). By contrast, PAR1 IgG or BMS200261 markedly inhibited platelet aggregation in response to 1nM thrombin, as did prior desensitization of platelets with the PAR1 agonist SFLLRN (Fig. 6). None of these maneuvers inhibited platelet aggregation in response to GYPGKF or submaximal concentrations of adenosine diphosphate (ADP) (Fig. 6 and data not shown). These data suggest that PAR1 is the major mediator of platelet activation at low concentrations of thrombin, consistent with previous studies (9, 25).

In contrast to the case at 1 nM thrombin, at 30 nM thrombin, inhibition of PAR1 signaling by either PAR1 IgG, antagonist, or SFLLRN desensitization was largely ineffective, only slowing aggregation slightly, such that shape change became detectable (see 0- to 30-second portions of

the aggregation curves in Fig. 6, *b–d*). Inhibition of PAR4 signaling with PAR4 IgG was similarly ineffective (Fig. 6*b*).

Strikingly, when signaling via PAR1 and PAR4 was blocked simultaneously, aggregation in response to even high concentrations of thrombin was virtually abolished (Fig. 6). Such synergy was seen regardless of the means by which PAR1 was blocked (desensitization, PAR1 IgG, or antagonist) (Fig. 6). PAR4 preimmune IgG had no effect in such experiments (data not shown), and platelet activation by ADP and by GYPGKF, which bypasses the effect of the PAR4 antibody, were not inhibited (Fig. 6, and data not shown). These data strongly suggest that both PAR1 and PAR4 contribute to platelet activation at high (30 nM) concentrations of thrombin and that inhibition of both receptors is required to ablate thrombin-triggered platelet aggregation.

Inhibition of thrombin-induced platelet secretion by blocking PAR1 and PAR4 signaling. A more quantitative measure of platelet activation is the amount and rate of adenosine triphosphate (ATP) release due to the secretion of platelet granule contents. We therefore measured the effect of BMS200261, PAR4 IgG, or both, on peak ATP release and the time to half-maximal ATP release in response to 30 nM thrombin (Fig. 7). PAR1 antagonism with BSM200261 decreased maximal ATP secretion to approximately one-third of control levels and prolonged the time to half-maximal secretion by approximately threefold. PAR1 IgG alone had a similar effect (not shown). PAR4 antibody alone had no effect on the tempo of ATP secretion and only a small effect, if any, on peak response. Strikingly, the combination of BMS200261 and PAR4 IgG ablated ATP secretion in response to 30 nM thrombin. Even after 10 minutes, no secretion was detected. Similar data were obtained when PAR1 IgG was combined with PAR4 IgG. These maneuvers did not block secretion in response to GYPGKF (Fig. 7), and preimmune and nonimmune IgG were without effect (data not shown). These results support the hypothesis that PAR1 and PAR4 account for platelet secretion and aggregation in response to thrombin at concentrations as high as 30 nM. They also suggest that PAR1 is necessary for rapid platelet activation by thrombin even at high thrombin concentrations.

Discussion

In this study, we addressed the roles of PAR1, PAR3, and PAR4 in activation of human platelets by thrombin. We showed that PAR1 and PAR4 are functionally expressed in human platelets and that these receptors account for most if not all thrombin signaling in these cells. We further demonstrate that PAR1 mediates platelet responses at low concentrations of thrombin and is necessary for the most rapid and robust platelet responses, even at high concentrations of thrombin, consistent with previous studies (9, 10). In contrast to PAR1, PAR4 mediates platelet activation only at high thrombin concentrations and PAR4 signaling appears unnecessary for platelet activation when PAR1 function is intact.

The observation that specific inhibition of PAR1 and PAR4 ablate thrombin signaling in human platelets suggests thrombin binding to GPIbα (28) is not sufficient to trigger platelet activation; whether such binding plays any adjunctive role is not known. Our results also sug-

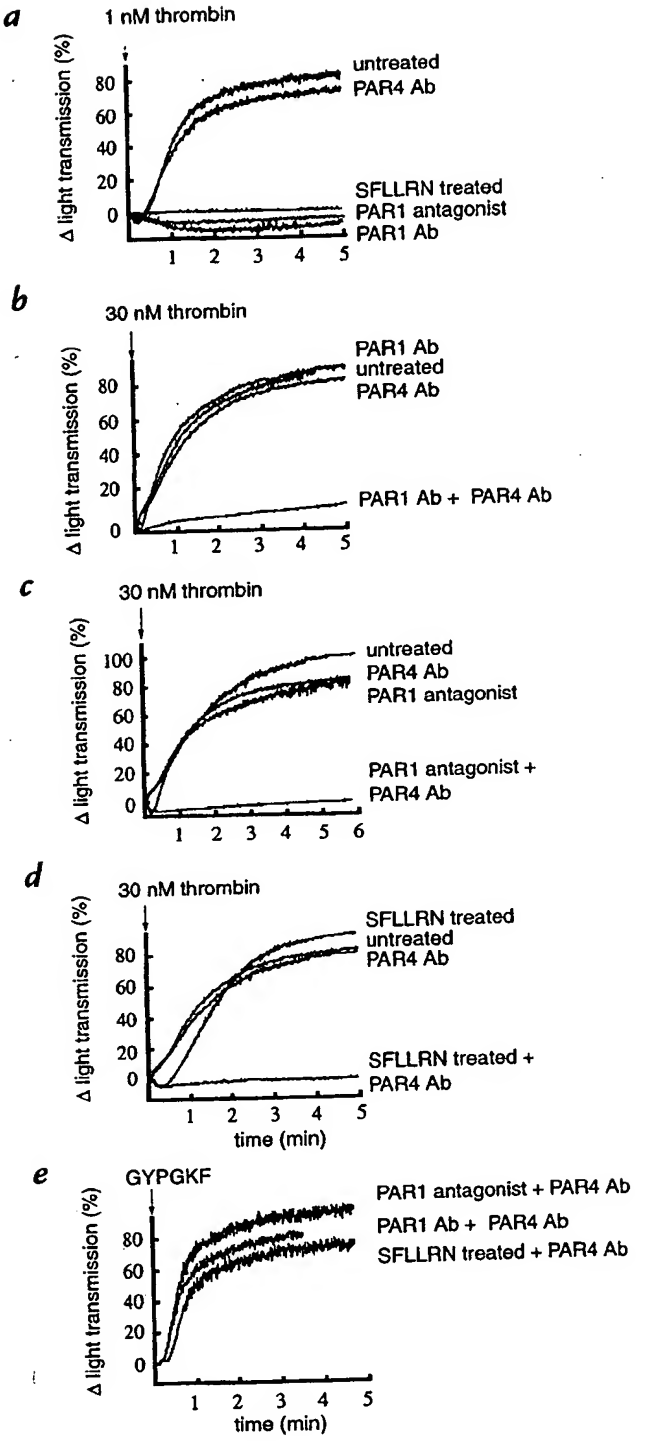


Figure 6
The effects of inhibition of PAR1 and/or PAR4 on aggregation of human platelets in response to low (1 nM) and high (30 nM) concentrations of thrombin. Platelets were pretreated with buffer alone, PAR1 IgG (10 μg/ml), PAR4 IgG (1 mg/ml), or PAR1 antagonist (100 μM), or were desensitized to SFLLRN as indicated and then exposed to 1 nM thrombin (*a*), 30 nM thrombin (*b–d*), or 500 μM GYPGKF (*e*) at time 0. Aggregation was measured as increase in light transmission. Preimmune or nonimmune IgG were without effect (not shown). This experiment was performed using triplicate samples twice (*a*, *c*, *e*) or four times (*b*, *d*). Representative tracings are shown.

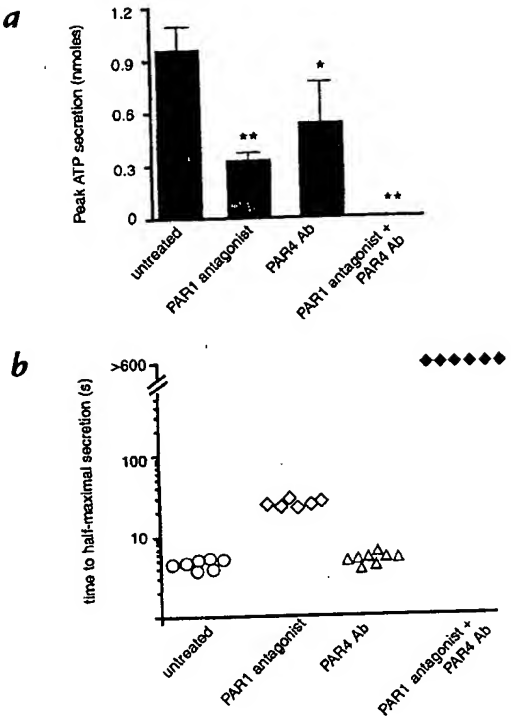


Figure 7
The effects of inhibition of PAR1 and/or PAR4 on platelet ATP secretion in response to thrombin. (*a*) Peak ATP secretion. Platelets were pretreated with buffer alone, PAR4 IgG (1 mg/ml), PAR1 antagonist BMS200261 (100 μM), or PAR1 antagonist plus PAR4 IgG as indicated, and then stimulated with 30 nM thrombin. Peak ATP concentration in the 10 min after addition of thrombin was measured by lumiaggregometry. Preimmune IgG had no effect (not shown). Data are mean ± SD (n = 5–7) of peak secretion measured; similar results were obtained with platelets from two individuals. Data were analyzed by two-way ANOVA and t test with a Bonferroni correction for multiple comparisons. $*P \cong 0.06$, $**P < 0.001$ compared with untreated group. Note that no secretion was detected during the 10 min after addition of 30 nM thrombin to platelets treated with PAR1 antagonist plus PAR4 IgG. (*b*) Time to half-maximal secretion. Time to reach 50% of the peak ATP secretion response elicited by 30 nM thrombin in each group (*a*) was measured. Platelets were pretreated with buffer alone (*open circles*), PAR1 antagonist (*open diamonds*), PAR4 IgG (*open triangles*), or PAR1 antagonist plus PAR4 Ab (*closed diamonds*) as in *a*, and then stimulated with 30 nM thrombin. Points displayed as >600 s indicate no measurable secretion within 10 min after addition of thrombin. PAR4 preimmune IgG had no effect inhibitory effect in such experiments, even in the presence of PAR1 antagonist (not shown).

gest that PAR3 has no important role in activation of human platelets by thrombin. Indeed, PAR3 mRNA and protein were not detected in human platelets but were readily detected in Dami cells. Such negative data regarding PAR3 expression in platelets must of course be interpreted with caution. Our failure to detect PAR3 mRNA and protein in human platelets is concordant with our functional data but at variance with a recent report of PAR3 expression in human platelets detected by RT-PCR and flow cytometry (26). In the latter study, the RT-PCR was not quantitative, and potential cross-reactivity of the PAR3 antiserum was not discussed, possibly accounting for our different results. Alternatively, platelets from only a handful of individuals have been studied, and it is possible that regulation or individual differences in PAR3 expression account for our differing results. Regardless, we know of no data that implicate PAR3 function in activation of human platelets by thrombin.

It is interesting to compare and contrast thrombin signaling in human and mouse platelets. This study shows that human platelets utilize both PAR1 and PAR4, with no apparent role for PAR3. By contrast, mouse platelets utilize PAR3 and PAR4 (17), with no apparent role for PAR1 (14). A definitive answer to whether additional receptors play a role in the mouse awaits generation of PAR3/PAR4 double knockout mice. In human platelets, PAR1 is necessary for responses to low concentrations of thrombin; in mouse platelets, PAR3 plays this role (17). Thus, despite the use of distinct receptors, platelets in both species use a dual thrombin receptor system in which a high-affinity receptor (PAR1 in human, PAR3 in mouse) mediates responses to low concentrations of thrombin and a low-affinity receptor (PAR4) mediates signaling at high concentrations. Interestingly, both PAR1 and PAR3 have obvious hirudin-like domains (3, 29). In PAR1, this domain binds thrombin's fibrinogen binding exosite and is critical for PAR1's efficient cleavage and activation by thrombin (3, 5, 29–32). PAR4 has no such domain, perhaps accounting for its slower cleavage by thrombin and right-shifted concentration response curve (17, 18).

The biological significance of having dual thrombin receptors in platelets remains uncertain. PAR3-deficient mice showed no spontaneous bleeding and had normal bleeding times; thus, in mice, the high-affinity receptor is not necessary for normal hemostasis when the low-affinity receptor is present. Whether different challenges will unmask a requirement for PAR3 in hemostasis or thrombosis is unknown, and whether combined deficiency in PAR3 and PAR4 will provoke a bleeding diathesis remains to be determined. It is possible that two receptors in platelets simply provide redundancy in an important system, but a variety of more interesting possibilities are apparent. It is possible that a capacity to respond to thrombin over a greater concentration range is important for reasons not yet understood. More broadly, PAR1, PAR3, and PAR4 might mediate responses to proteases or ligands other than thrombin or allow thrombin itself to activate distinct signaling pathways or to trigger signaling with varied tempos of activation or shutoff. The existence of multiple receptors also allows for distinct temporal and spatial expression. The finding of PAR3 expression in Dami cells (Figs. 1 and 2), but not in human platelets, is provocative in this regard. Dami cells were derived from a patient with megakaryoblastic leukemia and express a number of megakaryocyte markers (19). PAR3 is also expressed by HEL cells and K562 cells (data not shown, and ref. 26). These results raise the possibility that PAR3 might be expressed by hematopoietic cells in the erythroid/megakaryocyte lineage but extinguished in mature megakaryocytes and platelets. The role of PAR expression in hematopoiesis, if any, and whether PARs might serve as useful markers of differentiation, remains to be explored.

Because of the role of thrombin and platelet activation in myocardial infarction and other pathological processes, identifying and blocking the receptors by which thrombin

activates platelets has been an important goal. Iterations around PAR1's tethered ligand sequence SFLLRN have already led to the development of potent peptide-based antagonists (27). These antagonists blocked human platelet activation by SFLLRN itself and by low concentrations of thrombin but were ineffective at high concentrations of thrombin (27). This study strongly suggests that persistent platelet responses to high thrombin concentrations in the presence of a PAR1 antagonist were due to PAR4, which is not blocked by the antagonist. The PAR1 antagonist BMS200261 was in fact quite effective at blocking activation of PAR1 by high concentrations of thrombin (Fig. 5, and data not shown) but became effective at blocking platelet activation by high concentrations of thrombin only when PAR4 was blocked simultaneously.

The finding that PAR1 and PAR4 account for all, or virtually all, of the ability of human platelets to respond to thrombin should excite interest in the development of thrombin receptor antagonists as possible antithrombotic agents. Agents that inhibit signaling via the thromboxane and ADP receptors are effective antithrombotic drugs (33, 34). Given thrombin's remarkable potency as a platelet activator and its ability to activate even aspirin-treated platelets (35, 36), blockade of thrombin signaling in platelets might also prove to be a useful strategy for preventing thrombosis. Because inhibition of PAR1 alone markedly attenuated platelet responses at low concentrations of thrombin, PAR1 antagonism might be sufficient for an antithrombotic effect. In this scenario, PAR4 might ensure a minimal level of thrombin signaling and act as a safety buffer. Alternatively, it may be necessary to block both PAR1 and PAR4 to prevent thrombosis. Using genetically modified mice and inhibitor studies in other species, it will now be possible to determine if these strategies should be pursued.

Acknowledgments

We thank Sayuri Satoh for technical assistance, and Hideyuki Kanno for synthesis of BMS200261. M.L. Kahn was supported by National Institutes of Health (NIH) grant K08 HL-03731 and by a Howard Hughes Postdoctoral Fellowship. M.J. Shapiro was supported by a postdoctoral fellowship from the American Heart Association, Western States Affiliate. This work was supported in part by NIH grants HL-44907 and HL-59202.

1. Davey, M., and Luscher, E. 1967. Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature.* **216**:857–858.
2. Berndt, M., and Phillips, D. 1981. Platelet membrane proteins: composition and receptor function. In *Platelets in biology and pathology.* J.L. Gordon, editor. Elsevier. Amsterdam, the Netherlands. 43–74.
3. Vu, T.-K.H., Hung, D.T., Wheaton, V.I., and Coughlin, S.R. 1991. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell.* **64**:1057–1068.
4. Rasmussen, U.B., *et al.* 1991. cDNA cloning and expression of a hamster alpha-thrombin receptor coupled to Ca2+ mobilization. *FEBS Lett.* **288**:123–128.
5. Vu, T.-K.H., Wheaton, V.I., Hung, D.T., and Coughlin, S.R. 1991. Domains specifying thrombin-receptor interaction. *Nature.* **353**:674–677.
6. Chen, J., Ishii, M., Wang, L., Ishii, K., and Coughlin, S.R. 1994. Thrombin receptor activation: confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. *J. Biol. Chem.* **269**:16041–16045.
7. Vassallo, R.J., Kieber, E.T., Cichowski, K., and Brass, L.F. 1992. Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.* **267**:6081–6085.
8. Scarborough, R.M., *et al.* 1992. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J. Biol. Chem.* **267**:13146–13149.
9. Hung, D.T., Vu, T.-K.H., Wheaton, V.I., Ishii, K., and Coughlin, S.R. 1992. Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *J. Clin. Invest.* **89**:1350–1353.
10. Brass, L.F., *et al.* 1992. Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J. Biol. Chem.* **267**:13795–13798.
11. Molino, M., Bainton, D.F., Hoxie, J.A., Coughlin, S.R., and Brass, L.F. 1997. Thrombin receptors on human platelets. Initial localization and subsequent redistribution during platelet activation. *J. Biol. Chem.* **272**:6011–6017.
12. Derian, C.K., Santulli, R.J., Tomko, K.A., Haertlein, B.J., and Andrade-Gordon, P. 1995. Species differences in platelet responses to thrombin and SFLLRN. Receptor-mediated calcium mobilization and aggregation and regulation by protein kinases. *Thromb. Res.* **6**:505–519.
13. Connolly, T.M., *et al.* 1994. Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thromb. Haemost.* **72**:627–633.
14. Connolly, A.J., Ishihara, H., Kahn, M.L., Farese, R.V., and Coughlin, S.R. 1996. Role of the thrombin receptor in development and evidence for a second receptor. *Nature.* **381**:516–519.
15. Ishihara, H., *et al.* 1997. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature.* **386**:502–506.
16. Ishihara, H., Zeng, D., Connolly, A.J., Tam, C., and Coughlin, S.R. 1998. Antibodies to protease-activated receptor 3 inhibit activation of mouse platelets by thrombin. *Blood.* **91**:4152–4157.
17. Kahn, M.L., *et al.* 1998. A dual thrombin receptor system for platelet activation. *Nature.* **394**:690–694.
18. Xu, W.F., *et al.* 1998. Cloning and characterization of human protease-activated receptor 4. *Proc. Natl. Acad. Sci. USA.* **95**:6642–6646.
19. Greenberg, S.M., Rosenthal, D.S., Greeley, T.A., Tantravahi, R., and Handin, R.I. 1988. Characterization of a new megakaryocytic cell line: the Dami cell. *Blood.* **72**:1968–1977.
20. Ishii, K., Hein, L., Kobilka, B., and Coughlin, S.R. 1993. Kinetics of thrombin receptor cleavage on intact cells. Relation to signaling. *J. Biol. Chem.* **268**:9780–9786.
21. Williams, J.A., McChesney, D.J., Calayag, M.C., Lingappa, V.R., and Logsdon, C.D. 1988. Expression of receptors for cholecystokinin and other Ca2+-mobilizing hormones in *Xenopus* oocytes. *Proc. Natl. Acad. Sci. USA.* **85**:4939–4943.
22. Shapiro, M.J., Trejo, J., Zeng, D.W., and Coughlin, S.R. 1996. Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking – distinct determinants for agonist-triggered versus tonic internalization and intracellular localization. *J. Biol. Chem.* **271**:32874–32880.
23. Trejo, J., Connolly, A.J., and Coughlin, S.R. 1996. The cloned thrombin receptor is necessary and sufficient for activation of mitogen-activated protein kinase and mitogenesis in mouse lung fibroblasts. Loss of responses in fibroblasts from receptor knockout mice. *J. Biol. Chem.* **271**:21536–21541.
24. Howells, G.L., *et al.* 1997. Proteinase-activated receptor-2: expression by human neutrophils. *J. Cell Sci.* **110**:881–887.
25. Brass, L.F., *et al.* 1992. Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J. Biol. Chem.* **267**:13795–13798.
26. Schmidt, V.A., *et al.* 1998. The human proteinase-activated receptor-3 (PAR-3) gene. Identification within a Par gene cluster and characterization in vascular endothelial cells and platelets. *J. Biol. Chem.* **273**:15061–15068.
27. Bernatowicz, M.S., *et al.* 1996. Development of potent thrombin receptor antagonist peptides. *J. Med. Chem.* **39**:4879–4887.
28. Okamura, T., Hasitz, M., and Jamieson, G.A. 1978. Platelet glycocalicin: interaction with thrombin and role as thrombin receptor on the platelet surface. *J. Biol. Chem.* **253**:3435–3443.
29. Ishihara, H., *et al.* 1997. Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature.* **386**:502–506.
30. Liu, L., Vu, T.-K.H., Esmon, C.T., and Coughlin, S.R. 1991. The region of the thrombin receptor resembling hirudin binds to thrombin and alters enzyme specificity. *J. Biol. Chem.* **266**:16977–16980.
31. Mathews, I.I., *et al.* 1994. Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. *Biochemistry.* **33**:3266–3279.
32. Ishii, K., Gerszten, R., Zheng, Y.-W., Turck, C.W., and Coughlin, S.R. 1995. Determinants of thrombin receptor cleavage: receptor domains involved, specificity, and role of the P3 aspartate. *J. Biol. Chem.* **270**:16435–16440.
33. Patrono, C. 1994. Aspirin as an antiplatelet drug. *N. Engl. J. Med.* **330**:1287–1294.
34. Balsano, F., *et al.* 1990. Antiplatelet treatment with ticlopidine in unstable angina. A controlled multicenter clinical trial. The Studio della Ticlopidina nell'Angina Instabile Group. *Circulation.* **82**:17–26.
35. Evans, G., Packham, M.A., Nishizawa, E.E., Mustard, J.F., and Murphy, E.A. 1968. The effect of acetylsalicylic acid on platelet function. *J. Exp. Med.* **128**:877–894.
36. Smith, J.B., and Willis, A.L. 1971. Aspirin selectively inhibits prostaglandin production in human platelets. *Nature New Biol.* **231**:235–237.

were moved close to a perfusion pipette containing standard extracellular solution supplemented with Cu(II)(1,10-phenanthroline)$_3$ at different concentrations. A Cu(II)(1,10-phenanthroline)$_3$ stock solution was made by dissolving 150 mM $CuSO_4$ and 500 mM 1,10 phenanthroline in 4:1 water/ethanol. After reaching steady-state conditions, cells were moved to another perfusion pipette with standard extracellular solution containing 1 mM DTT. Current modifications under oxidizing and reducing conditions were observed as described for the MTS experiments. The time course was fit with a single exponential giving the time constant of modification.

To determine block by intracellular Cd^{2+}, Cd^{2+} was added in variable concentration to a modified intracellular solution devoid of EGTA containing (in mM): NaCl 140, $MgCl_2$ 2, HEPES 10, pH 7.4. Initially, the voltage dependence of the instantaneous current amplitude after a 300-ms prepulse to +75 mV was obtained on inside-out patches in standard intracellular solution. Then the Cd^{2+}-containing solution was applied by moving the patch into the stream of a perfusion pipette. The time course of the block was determined by repetitive pulsing. After reaching a steady-state value, the voltage dependence of the instantaneous current amplitudes was measured with the patch pipette in the solution stream and shortly after switching to standard intracellular solution. Relative block was obtained by dividing the current amplitude at the end of the prepulse before and after Cd^{2+} application.

Data were analysed with a combination of PClamp (Axon Instruments) and SigmaPlot (Jandel Scientific) programs. All data are shown as means ± s.e.m.

Received 20 February; accepted 5 June 1998.

1. Jentsch, T. J. Molecular biology of voltage-gated chloride channels. *Curr. Top. Membr.* **42**, 35–57 (1994).
2. Miller, C. Open-state substructure of single chloride channels from *Torpedo* electroplax. *Phil. Trans. R. Soc. Lond. B* **299**, 401–411 (1982).
3. Middleton, R. E., Pheasant, D. J. & Miller, C. Homodimeric architecture of a ClC-type chloride ion channel. *Nature* **383**, 337–340 (1996).
4. Ludewig, U., Pusch, M. & Jentsch, T. J. Two physically distinct pores in the dimeric ClC-0 chloride channel. *Nature* **383**, 340–343 (1996).
5. Malinowska, D. H., Kupert, E. Y., Bahinski, A., Sherry, A. M. & Cuppoletti, J. Cloning, functional expression, and characterization of a PKA-activated gastric Cl^- channel. *Am. J. Physiol. Cell Physiol.* **268**, C191–C200 (1995).
6. Duan, D., Winter, C., Cowley, S., Hume, J. R. & Horowitz, B. Molecular identification of a volume-regulated chloride channel. *Nature* **390**, 417–421 (1997).
7. Fahlke, Ch., Yu, H. T., Beck, C. L., Rhodes, T. H. & George, A. L. Jr Pore-forming segments in voltage-gated chloride channels. *Nature* **390**, 529–532 (1997).
8. Middleton, R. E., Pheasant, D. J. & Miller, C. Purification, reconstitution, and subunit composition of a voltage-gated chloride channel from *Torpedo* electroplax. *Biochemistry* **33**, 13189–13198 (1994).
9. Fahlke, Ch., Knittle, T. J., Gurnett, C. A., Campbell, K. P. & George, A. L. Jr Subunit stoichiometry of human muscle chloride channels. *J. Gen. Physiol.* **109**, 93–104 (1997).
10. Roberts, D. D., Lewis, S. D., Ballou, D. P., Olson, S. T. & Shafer, J. A. Reactivity of small thiolate anions and cysteine-25 in papain toward methyl methanethiosulfate. *Biochemistry* **25**, 5595–5601 (1986).
11. Fersht, A. R. *Enzyme Structure and Mechanism* (W. H. Freeman, New York, 1985).
12. Cheung, M. & Akabas, M. H. Locating the anion-selectivity filter of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. *J. Gen. Physiol.* **109**, 289–299 (1997).
13. Careaga, C. L. & Falke, J. J. Thermal motions of surface alpha-helices in the D-galactose chemosensory receptor. Detection by disulfide trapping. *J. Mol. Biol.* **226**, 1219–1235 (1992).
14. Benitah, J. *et al.* Molecular motions within the pore of voltage-dependent sodium channels. *Biophys. J.* **73**, 603–613 (1997).
15. Glusker, J. P. Structural aspects of metal liganding to functional groups in proteins. *Adv. Protein Chem.* **42**, 1–76 (1991).
16. Benitah, J., Tomaselli, G. F. & Marban, E. Adjacent pore-lining residues within sodium channels identified by paired cysteine mutagenesis. *Proc. Natl Acad. Sci. USA* **93**, 7392–7396 (1996).
17. Rychkov, G. Y. *et al.* pH-dependent interactions of Cd^{2+} and a carboxylate blocker with the rat ClC-1 chloride channel and its R304E mutant in the Sf-9 insect cell line. *J. Physiol. (Lond.)* **501**, 355–362 (1997).
18. Heginbotham, L. & MacKinnon, R. The aromatic binding site for tetraethylammonium ion on potassium channels. *Neuron* **8**, 483–491 (1992).
19. Carter, P. J., Winter, G., Wilkinson, A. J. & Fersht, A. R. The use of double mutants to detect structural changes in the active site of the tyrosyl-tRNA synthetase (*Bacillus stearothermophilius*). *Cell* **38**, 835–840 (1984).
20. Hidalgo, P. & MacKinnon, R. Revealing the architecture of a K^+ channel pore through mutant cycles with a peptide inhibitor. *Science* **268**, 307–310 (1995).
21. Root, M. J. & MacKinnon, R. Two identical noninteracting sites in an ion channel revealed by proton transfer. *Science* **265**, 1852–1856 (1994).
22. Li, M., Unwin, N., Stauffer, K. A., Jan, Y. N. & Jan, L. Y. Images of purified *Shaker* potassium channels. *Curr. Biol.* **4**, 110–115 (1994).
23. Doyle, D. A. *et al.* The structure of the potassium channel: Molecular basis of K^+ conduction and selectivity. *Science* **280**, 69–77 (1998).
24. Fahlke, Ch., Beck, C. L. & George, A. L. Jr A mutation in autosomal dominant myotonia congenita affects pore properties of the muscle chloride channel. *Proc. Natl Acad. Sci. USA* **94**, 2729–2734 (1997).

Acknowledgements. We thank L. DeFelice, P. Hidalgo, M. Holmgren, R. Horn, J. P. Johnson, N. Mitrovic and C. I. Petersen for discussions and review of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft (Ch.F.), the Muscular Dystrophy Association (Ch.F., A.L.G.) and the National Institutes of Health (A.L.G.).

Correspondence and request for materials should be addressed to Ch.F. (e-mail: christoph.fahlke@mcmail.vanderbilt.edu).

A dual thrombin receptor system for platelet activation

Mark L. Kahn*†, Yao-Wu Zheng*, Wei Huang*, Violeta Bigornia*, Dewan Zeng*, Stephen Moff*, Robert V. Farese Jr†‡, Carmen Tam* & Shaun R. Coughlin*†§

** Cardiovascular Research Institute and Daiichi Research Center, † Department of Medicine, ‡ Gladstone Institute of Cardiovascular Disease, and § Department of Cellular and Molecular Pharmacology, University of California, Box 0130, 505 Parnassus Avenue, San Francisco, California 94143-0130, USA*

Platelet-dependent arterial thrombosis triggers most heart attacks and strokes. Because the coagulation protease thrombin is the most potent activator of platelets[1], identification of the platelet receptors for thrombin is critical for understanding thrombosis and haemostasis. Protease-activated receptor-1 (PAR1) is important for activation of human platelets by thrombin[2–6], but plays no apparent role in mouse platelet activation[7–9]. PAR3 is a thrombin receptor that is expressed in mouse megakaryocytes[10]. Here we report that thrombin responses in platelets from PAR3-deficient mice were markedly delayed and diminished but not absent. We have also identified PAR4, a new thrombin-activated receptor. PAR4 messenger RNA was detected in mouse megakaryocytes and a PAR4-activating peptide caused secretion and aggregation of PAR3-deficient mouse platelets. Thus PAR3 is necessary for normal thrombin responses in mouse platelets, but a second PAR4-mediated mechanism for thrombin signalling exists. Studies with PAR-activating peptides suggest that PAR4 also functions in human platelets, which implies that an analogous dual-receptor system also operates in humans. The identification of a two-receptor system for platelet activation by thrombin has important implications for the development of antithrombotic therapies.

PAR3-deficient mice (Fig. 1) developed normally and showed no spontaneous bleeding. Haematocrit, platelet counts and bleeding times[11] were indistinguishable from those of wild-type mice (data not shown). However, thrombin responses in PAR3-deficient platelets were markedly abnormal (Fig. 2). Wild-type platelets secreted their granule contents and aggregated reliably to 1 nM thrombin. In contrast, PAR3-deficient platelets were virtually unresponsive to 1 and 3 nM thrombin; 10 nM thrombin elicited delayed responses from PAR3-deficient platelets (Fig. 2) and the level of secretion eventually achieved by PAR3-deficient platelets was decreased compared with that in wild-type platelets. Even at 30 nM thrombin, the secretion response in PAR3-deficient platelets was delayed, but the level of secretion ultimately reached was comparable to that seen in wild-type platelets (Fig. 2). Secretion and aggregation in response to U46619, an agonist of the thromboxane receptor, were indistinguishable in wild-type and PAR3-deficient platelets even at submaximal agonist concentrations (data not shown). Thus PAR3 is necessary for normal responsiveness to thrombin in mouse platelets.

However diminished, thrombin responses did persist in PAR3-deficient platelets. What mediates these responses? The prototypical thrombin receptor PAR1 (ref. 2) plays no role in activation of mouse platelets by thrombin[9], and PAR3-deficient platelets, like wild-type platelets[7–9], did not respond to PAR1-activating peptide (data not shown). Persistent thrombin signalling in PAR3-deficient platelets therefore suggested the presence of an as yet uncharacterized thrombin receptor—perhaps another PAR family member. A GenBank BLAST search for PAR-related sequences revealed an EST (for expressed sequence tag) encoding an 11-amino-acid sequence that was 73% identical to the cognate sequence in PAR2. Because this region is conserved among PARs, a full-length complementary DNA was obtained. This cDNA (GenBank accession

number AF080215) encoded a 397-amino-acid protein, now designated PAR4, that was most closely related to human PAR3, with 30% amino-acid sequence identity. Proteases activate PARs by cleaving their amino-terminal exodomains to unmask a new amino terminus that then serves as a tethered ligand, binding intramolecularly to the body of the receptor to effect transmembrane signalling[2,12,13]. Examination of PAR4's amino-terminal exodomain revealed a putative thrombin cleavage site (Fig. 3) that was identical to the thrombin cleavage site in rat PAR1 (ref. 14). Expression of PAR4 in *Xenopus* oocytes did confer robust signalling to thrombin, but, at least in this system, PAR4 required higher thrombin concentrations than did the well-studied thrombin receptor PAR1 (Fig. 3). Thrombin cleaved a Flag epitope fused to PAR4's amino terminus[15] from the surface of PAR4-expressing oocytes, and active site-inhibited PPACK thrombin[16] did not cause cleavage or signalling (data not shown), which is consistent with a proteolytic activation mechanism. PAR4 signalling was relatively thrombin-specific; among the related arginine/lysine-specific proteases tested, only thrombin and trypsin elicited significant responses (Fig. 3).

Synthetic peptides that mimic the tethered ligands of PAR1 and PAR2 function as agonists for their respective receptors and have been used as pharmacological tools to probe the function of these receptors in various cell types[2,5,6]; the cognate peptide for PAR3 appears to be insufficiently active to function as a free ligand[10]. The location of the putative thrombin cleavage site in PAR4 predicted that the sequence GYPGKF would serve as PAR4's tethered ligand, implying that a synthetic peptide of the same sequence might function as a PAR4 agonist. As predicted, this peptide activated PAR4-expressing *Xenopus* oocytes but not uninjected or water-injected oocytes (Fig. 3 and data not shown). In the thrombin receptors PAR1 and PAR3, the phenylalanine in the second position of the tethered ligand (a tyrosine in the PAR4-activating peptide) is critical for the function of this domain[5,6,10]. Accordingly, the synthetic peptide GAPGKF had no activity at PAR4 (Fig. 3).

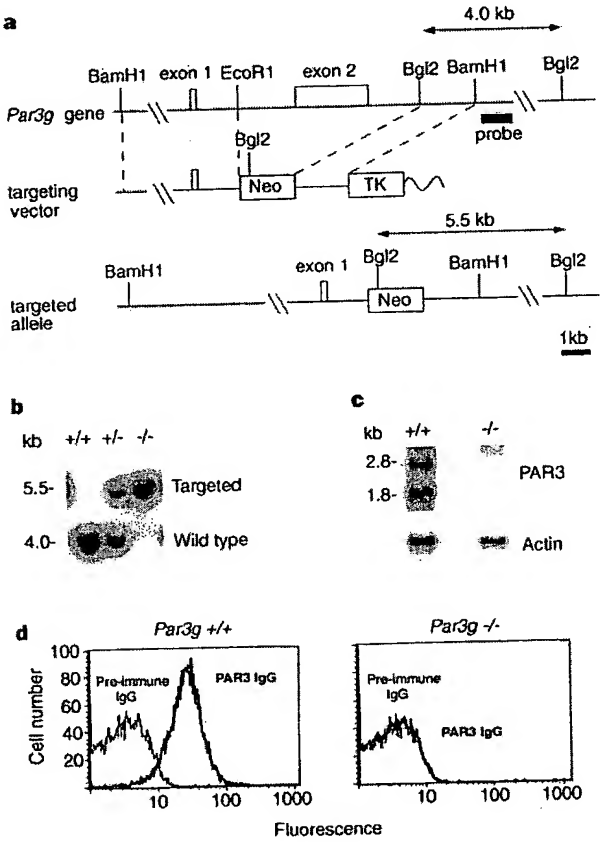


Figure 1 Generation of PAR3-deficient mice. **a**, Gene-targeting strategy. A replacement vector[25] was used to substitute a neomycin phosphotransferase expression cassette (Neo) for *Par3g* exon 2, which encodes the entire PAR3 protein except for its signal peptide. Wavy line represents plasmid backbone; TK, HSV thymidine kinase expression cassette. **b**, Southern-blot analysis of *Bgl*II-digested genomic DNA from the tails of pups derived from $Par3g^{+/-}$ matings using 3′ flanking probe (**a**). Targeting removed an endogenous *Bgl*II site and introduced a new *Bgl*II site. The 4.0-kb and 5.5-kb bands correspond to wild-type and targeted alleles, respectively. **c**, Northern-blot analysis of $Par3g^{+/+}$ and $Par3g^{-/-}$ mouse spleen mRNA using *Par3g* exon 2 probe and β-actin probe to control for lane loading. **d**, Flow cytometric analysis of wild-type and $Par3g^{-/-}$ platelets for PAR3 protein. Platelets were incubated with preimmune IgG (narrow line) or PAR3 IgG (wide line). Bound IgG was detected with FITC-labelled 2° antibody[23]. Each figure represents an analysis of 10,000 events. Note lack of surface PAR3 in $Par3g^{-/-}$ platelets.

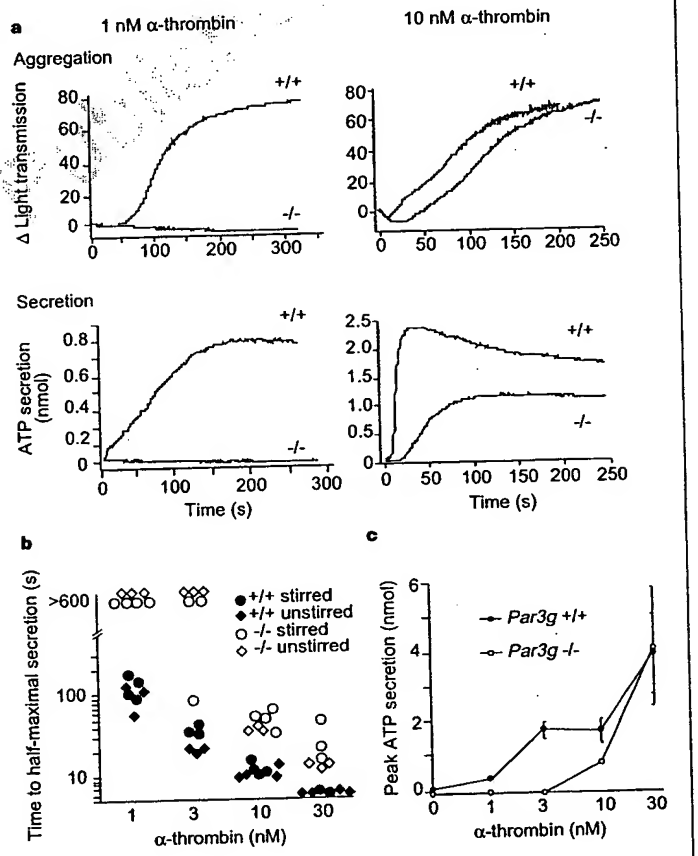


Figure 2 Thrombin responses in PAR3-deficient platelets. **a**, Aggregation and secretion of wild-type ($Par3g^{+/+}$) and PAR3-deficient ($Par3g^{-/-}$) platelets to 1 nM (left) and 10 nM (right) α-thrombin. Platelets were stirred and exposed to thrombin at 0 s. Similar results were obtained with five separate platelet preparations. **b**, Time to half-maximal secretion. $Par3g^{+/+}$ (filled symbols) and $Par3g^{-/-}$ (open symbols) platelets were stirred (circles) or left unstirred (diamonds) in a lumiaggregometer. Time to 50% of the peak response elicited by each of the indicated concentrations of thrombin was measured. Unstirred platelets did not aggregate. At each thrombin concentration, each point represents the response of a separate platelet preparation. Points at >600 s had no measurable secretion at 10 min. **c**, Dose response of $Par3g^{+/+}$ (filled circles) and $Par3g^{-/-}$ (open circles) platelets. Unstirred platelets were exposed to various concentrations of thrombin and peak ATP secretion was measured. Values are the mean responses (± s.e.m.) of three separate platelet preparations. Some error bars are obscured by the symbols.

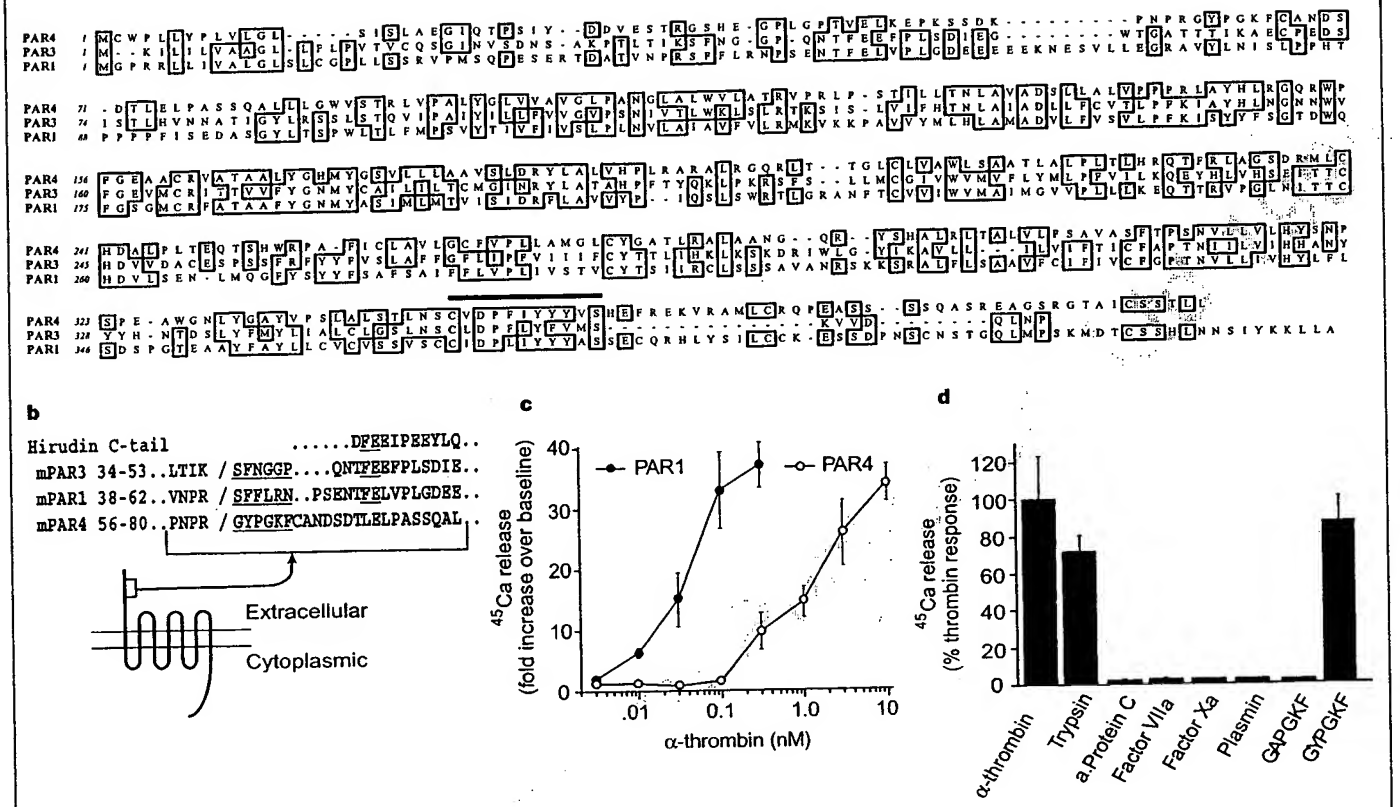


Figure 3 PAR4 amino-acid sequence and signalling properties. **a**, Sequence of PAR4 aligned with PAR3 and PAR1, the two known mouse thrombin receptors. Regions of amino-acid identity are boxed. The 11 amino acids of EST 400689 are overlined. **b**, Features of the amino-terminal exodomains of mouse thrombin receptors. The predicted thrombin cleavage site in PAR4 (/) is aligned with the known sites in PARs 1 and 3. The predicted or known tethered ligand sequences that are unmasked by receptor cleavage are underlined, as are amino-acid sequences resembling hirudin's carboxyl tail. The latter sequence in human PAR1 binds to thrombin's fibrinogen-binding exosite in a similar way to hirudin's C-tail[12,26,27]. **c**, Thrombin activation of PAR4 and PAR1. Agonist-triggered ^{45}Ca release was measured in *Xenopus* oocytes expressing mouse PAR4 and human PAR1 tagged at their amino termini with a FLAG epitope[15]. Data are mean ± s.e.m. ($n = 4$) and are expressed as the fold increase over baseline for each receptor. Surface expression of PAR4 measured with anti-FLAG monoclonal antibody was 1.8 times that of PAR1. Similar signalling data were obtained with untagged receptors. **d**, Specificity of PAR4 activation by proteases and peptides. ^{45}Ca release by oocytes expressing PAR4 was measured in response to the indicated active proteases (all at 5 nM except trypsin (0.5 nM)) or to the synthetic peptides GYPGKF and GAPGKF (each at 500 μM). Uninjected and buffer-injected oocytes did not respond to proteases or peptides at these concentrations. Data are mean ± s.e.m. ($n = 3$) and are expressed as a percentage of the maximum response to thrombin. In the experiment shown, thrombin caused a 33-fold increase in ^{45}Ca release. These experiments were replicated at least twice.

Taken together, these data show that PAR4 is a functional protease-activated receptor that is capable of mediating thrombin signalling.

Northern-blot analysis of mouse tissues revealed a relatively high level of expression of PAR4 in spleen, trace expression in heart, lung, skeletal muscle and kidney, and no detectable expression in brain, liver or testis (data not shown). *In situ* hybridization of spleen and bone marrow revealed that, like PAR3 (ref. 10), PAR4 mRNA is expressed in mouse megakaryocytes (Fig. 4). Finally, GYPGKF, a PAR4-activating peptide, activated both wild-type and PAR3-deficient mouse platelets, whereas the mutant GAPGKF peptide had no activity (Fig. 4 and data not shown). These data suggest that PAR4 functions in both wild-type and PAR3-deficient mouse platelets and is likely to be responsible for the residual thrombin signalling seen in the latter.

The characterization of a dual thrombin receptor system in mouse platelets leads to the question of whether a similar system operates in human platelets. Unlike mouse platelets, thrombin signalling in human platelets is mediated at least in part by PAR1 (refs 2–6, 12). A role for PAR3 in human platelets has not been demonstrated, but pharmacological studies have suggested that PAR1-independent mechanisms for activating human platelets may exist[3,17–20]. We have identified a human PAR4 cDNA (GenBank accession number AF080214) and studies using polymerase chain reaction (PCR) after reverse transcription of RNA suggest that it is expressed in human platelets (M.L.K., M. Nakanishi-Matsui and S.R.C., manuscript in preparation). In addition, both SFLLRN, a PAR1-activating peptide, and GYPGKF, a PAR4-activating peptide, activated human platelets; the mutant peptide GAPGKF did not (Fig. 4). The potency of these pharmacological tools for platelet activation correlated with their potency at their respective receptors in the oocyte system. GYPGKF activated human platelets at a threshold concentration of 300–500 μM and PAR4-expressing *Xenopus* oocytes with an EC_{50} (effector concentration for a half-maximal response) of 30 μM. SFLLRN activated human platelets at a threshold concentration of 1–3 μM and PAR1-expressing oocytes with an EC_{50} of 0.3 μM. SFLLRN showed no activity on PAR4-expressing oocytes and GYPGKF showed little activity on PAR1-expressing oocytes (<8% of maximal PAR1 activation even at 500 μM GYPGKF, data not shown). Lastly, GYPGKF activated PAR1-desensitized platelets (Fig. 4). These data suggest that PAR4 functions in human platelets.

In summary, PAR3 is necessary for normal thrombin signalling in mouse platelets but PAR4, a newly characterized thrombin receptor, also contributes. Human platelets also seem to use at least two

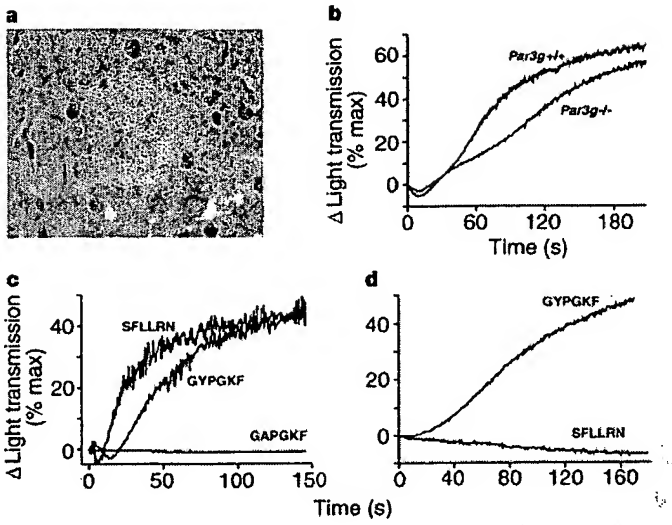


Figure 4 Expression of PAR4 in mouse megakaryocytes and evidence for PAR4 function in mouse and human platelets. **a**, *In situ* hybridization of mouse spleen for PAR4 mRNA. Bright-field photomicrograph shows silver grains overlying a megakaryocyte. Sense-probe controls were negative. **b**, GYPGKF, a PAR4-activating peptide, activates wild-type and PAR3-deficient mouse platelets. Stirred wild-type and PAR3-deficient platelets were exposed to GYPGKF (500 μM) at 0 s and aggregation was measured. Under the same conditions, no response to the control peptide GAPGKF was obtained (data not shown). This experiment was repeated three times. **c**, PAR4-activating peptide activates human platelets. Naive human platelets were exposed to the PAR1 agonist SFLLRN (3 μM), the PAR4 agonist GYPGKF (500 μM) or the mutant peptide GAPGKF (500 μM) and aggregation was measured. **d**, PAR4-activating peptide activates PAR1-desensitized human platelets. SFLLRN-desensitized platelets (see Methods) were exposed to either SFLLRN (500 μM) or GYPGKF (500 μM) and aggregation was measured. This experiment was replicated three times using platelets from two different donors. Note that GYPGKF activates naive and PAR1-desensitized human platelets.

receptors, PAR1 and PAR4, for thrombin signalling. Why might a two-receptor system for platelet activation by thrombin exist? It may simply provide redundancy in a pathway important for haemostasis. More interestingly, it may provide a mechanism for responding to proteases other than thrombin or to thrombin itself over a wider range of concentrations, for signalling with different tempos, for activating distinct downstream effectors, or for allowing differential regulation of receptor levels or function. The identification of this receptor system provides a framework for further defining the roles and relative importance of distinct PARs in platelets and other cells and for refining strategies for pharmaceutical development. For example, it may be necessary to block both PAR1 and PAR4 in human platelets to achieve an antithrombotic effect. Alternatively, the existence of a second receptor may provide a useful margin of safety for such potentially powerful therapeutic agents and/or the ability to block selected *in vivo* responses to thrombin.

Note added in proof: While this manuscript was in press, the identification of human PAR4 was reported independently[28]. □

Methods

Inactivation of the gene encoding PAR3 (*Par3g*). A bacterial artificial chromosome (BAC) that contained *Par3g* was obtained by PCR screen of a 129/SvJ mouse genomic library (Genome Systems). A 6.5-kilobase (kb) *Bam*H1/*Eco*RI fragment 5′ of exon 2 and a 2.0-kb *Bgl*II/*Bam*H1 fragment 3′ of exon 2 were cloned into the pNTK vector[21] to create the targeting vector (Fig. 1a). A 0.8-kb *Nde*I fragment of *Par3g* 3′ of the short arm of homology was used as a probe to identify both the wild-type and targeted alleles (Fig. 1a). RF8 ES cells[22] (129/SvJae) were electroporated with the targeting construct and clones that were resistant to G418 and FIAU were selected and screened by Southern blot. A highly chimaeric male mouse that was derived using $Par3g^{+/-}$ ES cells was bred to C57Bl/6 females to generate approximately 50 F_1 $Par3g^{+/-}$ mice. All experiments reported here were performed using the F_2 offspring of these mice.

Northern blot analysis. Poly(A)$^+$ RNA (3 μg) from wild-type and $Par3g^{-/-}$ mouse spleens was electrophoresed on a denaturing gel, transferred to reinforced nitrocellulose membrane (Schleicher & Schuell), and hybridized to a 600-base-pair (bp) *Eco*RI/*Sal*I PAR3 exon 2 probe under high stringency.

Flow cytometry. Washed mouse platelets were resuspended in platelet buffer (20 mM Tris-HCl pH 7.4, 140 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mg ml^{-1} glucose, 0.5% BSA, 1 μM PGE_1 and 5 mM EDTA), incubated with 10 μg ml^{-1} of anti-PAR3 IgG[23] at 4 °C for 1 h, washed, and then incubated with FITC-conjugated goat anti-rabbit IgG (Molecular Probes) at 4 μg ml^{-1} for 30 min. Platelets were then washed three times and analysed by flow cytometry.

Platelet aggregation and secretion. Blood was collected into citrate buffer from the inferior vena cava of pentobarbital-anaesthetized mice. Blood from 3–4 $Par3g^{-/-}$ mice or their wild-type littermates was pooled for each platelet study. Platelet-rich plasma was prepared by centrifugation of whole blood at 200 *g* for 7 min. EDTA (10 mM) and PGE_1 (1 μM) were then added and platelet-rich plasma was centrifuged at 500 *g* for 10 min. Platelets were then washed in platelet buffer containing 1 mM EDTA and 1 μM prostaglandin PGE_1, collected by centrifugation, resuspended to an OD_{500} of 1.0 (~2.5 × 10^8 platelets per millilitre) in platelet buffer lacking EDTA and PGE_1, and incubated on ice for 30 min before use. Calcium chloride was added to a final concentration of 2 mM and aggregation and secretion were measured in a Chrono-Log lumiaggregometer. Platelet suspension (300 μl) was added to the aggregometer chamber and change in light transmission after addition of agonist was followed. Results are expressed as the change (Δ) in light transmission, defined as the per cent increase in light transmission over that of the unactivated platelet suspension, with 100% representing light transmission of platelet buffer alone. Platelet ATP secretion was measured by adding luciferase (880 units per millilitre) and luciferin (8 μg ml^{-1}) to each sample; the luminescence generated by platelet-released ATP was compared with that of an ATP standard. Human blood was drawn from the antecubital vein; otherwise human platelets were prepared in a manner identical to mouse platelets. For PAR1-desensitization studies, human platelets that were resuspended from the first platelet pellet were incubated with 100 μM SFLLRN peptide at room temperature for 5 min without stirring, and then washed and resuspended as above.

Cloning of PAR4. Mouse EST 400689 was identified by BLAST search of the NCBI EST database using the coding region of human PAR2. The EST was sequenced and found to encode the carboxy-terminal 32 amino acids of a presumed G-protein-coupled receptor. A full-length cDNA was obtained by a combination of 5′ rapid amplification of cloned ends (RACE) using cDNA

from mouse embryo at day 14–15 (Marathon cDNA, Clonetech), and PCR of cDNA from a mouse brain endothelial cell line (bEND cells, W. Risau). A BAC mouse genomic clone was obtained from Genome Systems. The sequence of the cDNA (Fig. 3) and genomic clones were consistent. Human PAR4 was cloned from K562 mRNA by RT-PCR using primers based on mouse PAR4 sequence.

Functional studies in *Xenopus* oocytes. cDNA encoding Flag-epitope-tagged PAR4 (ref. 15) was constructed such that the Flag epitope was followed by amino-acid 18 of PAR4. Epitope-tagged and wild-type PAR4 cDNAs in pFROG[2] were transcribed *in vitro* and *Xenopus* oocytes were microinjected with 0.5–5.0 ng of PAR4 cRNA and 25 ng of PAR1 cRNA per oocyte. Agonist-triggered ^{45}Ca mobilization, a reflection of phosphoinositide hydrolysis in these cells, was measured[2,24].

***In situ* hybridization.** *In situ* hybridization[10] was done using sense or antisense ^{35}S-riboprobe transcribed from mouse PAR4 cDNA in pBluescript II SK. An 8-week exposure is shown.

Received 13 March; accepted 15 June 1998.

1. Davey, M. & Luscher, E. Actions of thrombin and other coagulant and proteolytic enzymes on blood platelets. *Nature* 216, 857–858 (1967).
2. Vu, T.-K. H., Hung, D. T., Wheaton, V. I. & Coughlin, S. R. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. *Cell* 64, 1057–1068 (1991).
3. Hung, D. T., Vu, T.-K. H., Wheaton, V. I., Ishii, K. & Coughlin, S. R. The cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. Blocking antiserum to the thrombin receptor's hirudin-like domain. *J. Clin. Invest.* 89, 1350–1353 (1992).
4. Brass, L. F. *et al.* Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. *J. Biol. Chem.* 267, 13795–13798 (1992).
5. Vassallo, R. J., Kieber, E. T., Cichowski, K. & Brass, L. F. Structure-function relationships in the activation of platelet thrombin receptors by receptor-derived peptides. *J. Biol. Chem.* 267, 6081–6085 (1992).
6. Scarborough, R. M. *et al.* Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J. Biol. Chem.* 267, 13146–13149 (1992).
7. Connolly, T. M. *et al.* Species variability in platelet and other cellular responsiveness to thrombin receptor-derived peptides. *Thromb. Hemost.* 72, 627–633 (1994).
8. Derian, C. K., Santulli, R. J., Tomko, K. A., Haertlein, B. J. & Andrade-Gordon, P. Species differences in platelet responses to thrombin and SFLLRN. Receptor-mediated calcium mobilization and aggregation and regulation by protein kinases. *Thromb. Res.* 6, 505–519 (1995).
9. Connolly, A. J., Ishihara, H., Kahn, M. L., Farese, R. V. & Coughlin, S. R. Role of the thrombin receptor in development and evidence for a second receptor. *Nature* 381, 516–519 (1996).
10. Ishihara, H. *et al.* Protease-activated receptor 3 is a second thrombin receptor in humans. *Nature* 386, 502–506 (1997).
11. Novak, E. K. *et al.* Cocoa: a new mouse model for platelet storage pool deficiency. *Br. J. Haematol.* 69, 371–378 (1988).
12. Vu, T.-K. H., Wheaton, V. I., Hung, D. T. & Coughlin, S. R. Domains specifying thrombin-receptor interaction. *Nature* 353, 674–677 (1991).
13. Chen, J., Ishii, M., Wang, L., Ishii, K. & Coughlin, S. R. Thrombin receptor activation: confirmation of the intramolecular tethered liganding hypothesis and discovery of an alternative intermolecular liganding mode. *J. Biol. Chem.* 269, 16041–16045 (1994).
14. Zhong, C., Hayzer, D. J., Corson, M. A. & Runge, M. S. Molecular cloning of the rat vascular smooth muscle thrombin receptor. Evidence for in vitro regulation by basic fibroblast growth factor. *J. Biol. Chem.* 267, 16975–16979 (1992).
15. Ishii, K., Hein, L., Kobilka, B. & Coughlin, S. R. Kinetics of thrombin receptor cleavage on intact cells: relation to signalling. *J. Biol. Chem.* 268, 9780–9786 (1993).
16. Kettner, C. & Shaw, E. D-phe-pro-argCH_2Cl: a selective affinity label for thrombin. *Thromb. Res.* 14, 969–973 (1979).
17. Shuman, M., Botney, M. & Fenton, J. I. Thrombin-induced platelet secretion. *J. Clin. Invest.* 63, 1211–1218 (1979).
18. Goodwin, C. A. *et al.* Thrombin receptor activating peptide does not stimulate platelet procoagulant activity. *Biochem. Biophys. Res. Commun.* 202, 321–327 (1994).
19. Kramer, R. M. *et al.* Differential activation of cytosolic phospholipase A2 (cPLA2) by thrombin and thrombin receptor agonist peptide in human platelets. Evidence for activation of cPLA2 independent of the mitogen-activated protein kinases ERK1/2. *J. Biol. Chem.* 270, 14816–14823 (1995).
20. Henriksen, R. A., Samokhin, G. P. & Tracy, P. B. Thrombin-induced thromboxane synthesis by human platelets. Properties of anion binding exosite I-independent receptor. *Arterioscler. Thromb. Vasc. Biol.* 17, 3519–3526 (1997).
21. Mortensen, R. in *Current Protocols in Molecular Biology* (ed. Ausubel, F. M.) 9.16.1 (Wiley, New York, 1993).
22. Meiner, V. L. *et al.* Disruption of the acyl-CoA:cholesterol acyltransferase gene in mice: evidence suggesting multiple cholesterol esterification enzymes in mammals. *Proc. Natl Acad. Sci. USA* 93, 14041–14046 (1996).
23. Ishihara, H., Zeng, D., Connolly, A. J., Tam, C. & Coughlin, S. R. Antibodies to protease-activated receptor 3 inhibit activation of mouse platelets by thrombin. *Blood* 91, 4152–4157 (1998).
24. Williams, J. A., McChesney, D. J., Calayag, M. C., Lingappa, V. R. & Logsdon, C. D. Expression of receptors for cholecystokinin and other Ca^{2+}-mobilizing hormones in Xenopus oocytes. *Proc. Natl Acad. Sci. USA* 85, 4939–4943 (1988).
25. Capecchi, M. R. Altering the genome by homologous recombination. *Science* 244, 1288–1292 (1989).
26. Liu, L., Vu, T.-K. H., Esmon, C. T. & Coughlin, S. R. The region of the thrombin receptor resembling hirudin binds to thrombin and alters enzyme specificity. *J. Biol. Chem.* 266, 16977–16980 (1991).
27. Mathews, I. I. *et al.* Crystallographic structures of thrombin complexed with thrombin receptor peptides: existence of expected and novel binding modes. *Biochemistry* 33, 3266–3279 (1994).
28. Xu, W. F. *et al.* Cloning and characterization of human protease-activated receptor 4. *Proc. Natl Acad. Sci. USA* 95, 6642–6646 (1998).

Acknowledgements. We thank T. Yu for blastocyst injection and H. Bourne for critical reading of this manuscript and S. E. Millar for assistance with figures.

Correspondence and requests for materials should be addressed to S.R.C. (e-mail: shaun_coughlin@quickmail.ucsf.edu).

A mutation in succinate dehydrogenase cytochrome *b* causes oxidative stress and ageing in nematodes

Naoaki Ishii*, Michihiko Fujii†, Philip S. Hartman‡, Michio Tsuda*, Kayo Yasuda*, Nanami Senoo-Matsuda*§, Sumino Yanase*, Dai Ayusawa† & Kenshi Suzuki*

* *Department of Molecular Life Science, Tokai University School of Medicine, Isehara, Kanagawa 259-1193, Japan*

† *Department of Biochemistry, Kihara Institute for Biological Research, Yokohama City University, Totsuka, Yokohama, Kanagawa 244-0813, Japan*

‡ *Department of Biology, Texas Christian University, Fort Worth, Texas 76129, USA*

§ *Tokyo Research Laboratory, Kyowa Hakko Kogyo Co. Ltd., Machida, Tokyo 194-8533, Japan*

Much attention has focused on the aetiology of oxidative damage in cellular and organismal ageing[1–4]. Especially toxic are the reactive oxygen byproducts of respiration and other biological processes[5]. A *mev-1(kn1)* mutant of *Caenorhabditis elegans* has been found to be hypersensitive to raised oxygen concentrations[6,7]. Unlike the wild type, its lifespan decreases dramatically as oxygen concentrations are increased from 1 to 60% (ref. 7). Strains bearing this mutation accumulate markers of ageing (such as fluorescent materials and protein carbonyls) faster than the wild type[8,9]. We show here that *mev-1* encodes a subunit of the enzyme succinate dehydrogenase cytochrome *b*, which is a component of complex II of the mitochondrial electron transport chain. We found that the ability of complex II to catalyse electron transport from succinate to ubiquinone is compromised in *mev-1* animals. This may cause an indirect increase in superoxide levels, which in turn leads to oxygen hypersensitivity and premature ageing. Our results indicate that *mev-1* governs the rate of ageing by modulating the cellular response to oxidative stress.

Three-factor crosses using visible genetic markers placed *mev-1* between *unc-50(e306)* and *unc-49(e382)* on chromosome III (Fig. 1). We tested cosmids from this region for their ability to rescue *mev-1* mutants from oxygen hypersensitivity after germline transformation[10]. Cosmid T07C4, but not C38H2, M03C11 or other cosmids, was able to rescue the *mev-1* mutant phenotype (Fig. 1). By testing various subclones from this cosmid, we identified a 5.6-kilobase (kb) fragment that also restored wild-type resistance (Figs 1, 2). Rescue was essentially complete with respect to both oxygen hypersensitivity (Fig. 2a) and premature ageing (Fig. 2b). This fragment includes an open reading frame containing the conceptual gene *cyt-1*, which is homologous to the bovine succinate dehydrogenase (SDH) cytochrome b_{560} (ref. 11; GenBank accession number L26545 for *C. elegans* and S74803 for bovine, respectively). We found that the *mev-1(kn1)* strain contained a missense mutation resulting in a glycine-to-glutamic acid substitution in *cyt-1* (Fig. 3). This mutation created a restriction fragment-length polymorphism that enabled the restriction enzyme *Mro*I to cleave wild-type DNA but not *mev-1* DNA at position 323. As predicted from the sequences, digestion by *Mro*I of the products of polymerase chain reaction with reverse transcription (RT-PCR) yielded two bands from wild type (N2), one band from *mev-1(kn1)* and three bands from transgenic animals (*kn1;knIs2*) (Fig. 1). This confirms that the wild-type *cyt-1* gene that we introduced into the *mev-1* strain was expressed and that rescue was achieved by this gene. We also confirmed that a 2.8-kb wild-type PCR product of this gene, including regions from 1,566 bases upstream and 537 bases downstream, rescued *mev-1* (Fig. 1).

Thrombosis and Haemostasis © F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 72 (4) 627–33 (1994)

Species Variability in Platelet and other Cellular Responsiveness to Thrombin Receptor-derived Peptides

Thomas M. Connolly[1], Cindra Condra[1], Dong-Mei Feng[3], Jacquelynn J. Cook[2], Marla T. Stranieri[2], Christopher F. Reilly[2], Ruth F. Nutt[3], Robert J. Gould[1]

From the Departments of [1]Biological Chemistry, [2]Pharmacology and [3]Medicinal Chemistry, Merck Research Laboratories, West Point, PA, USA

Summary

The aggregation of platelets from a variety of animal species in response to thrombin receptor-derived activating peptides was evaluated. A series of 14-(SFLLRNPNDKYEPF), 7-(SFLLRNP-NH_2), 6-(SFLLRN-HN_2) or 5-(SFLLR-NH_2) residue peptides, the structures of which were based on the deduced amino acid sequence of the human thrombin receptor, promoted full aggregation of platelets in plasma from humans, African Green and Rhesus monkeys, baboons and guinea pigs at 4–50 μM depending on the peptide used. Platelets in plasma from rabbit, dog, pig, and hamster underwent a shape change but failed to aggregate in response to these peptides over 3 log units of peptide up to 800 μM, despite being fully responsive to human thrombin. However, because the receptor peptides induced shape change in the platelets from these non-aggregating species, they apparently can activate some of the intracellular signaling system(s) usually initiated by thrombin in these platelets. In contrast, platelets from rats did not undergo shape change or aggregate in response to the peptides. A 7-residue receptor-derived peptide based on the deduced amino acid sequence of the clone of the hamster thrombin receptor (SFFLRNP-N_2) was nearly as efficacious as the corresponding human receptor-derived 7-residue peptide to promote aggregation of human platelets. However, the hamster peptide could not promote aggregation of hamster platelets in plasma at up to 800 μM peptide, while a shape change response was elicited. Platelets from rats, rabbits and pigs also did not aggregate in response to this peptide derived from the hamster thrombin receptor, but all species except the rat underwent a shape change. Longer 17-residue peptides derived from the sequences of the hamster or mouse thrombin receptors elicited aggregation of human platelets but no aggregation of the hamster platelets. In contrast, the human 14- and 5-residue and the hamster 7-residue thrombin receptor-derived peptides promoted mitogenesis of CCL39 cells, a hamster fibroblast cell line. Finally, the human 6-residue thrombin receptor-derived peptide promoted contraction of normal and de-endothelialized canine coronary artery rings, despite having no pro-aggregatory effect on canine platelets. Taken together, these results demonstrate that the thrombin receptor-derived peptides are able to mimic many, but not all, of the activating effect of thrombin in different tissues from multiple species. The heterogeneity of responsiveness to these peptides should be taken into account in future experiments designed to elucidate the mechanism of thrombin stimulation of platelets and other cells.

Correspondence to: Dr. Th. M. Connolly, Department of Biological Chemistry, Merck Research Laboratories, West Point, PA 19486, USA – FAX Number: +215 652 2589

Introduction

The serine protease thrombin is the most potent physiological activator of platelets known. It directly activates platelets and other cells via one or more cell surface receptor(s) (1). The cloning (2, 3) of a thrombin receptor was recently described. Based on its primary sequence and on its characteristics of activation this receptor belongs to the family of 7 transmembrane G-protein linked receptors. According to the novel mechanism of activation of the receptor proposed by Coughlin et al. (2, 4) thrombin cleaves the extracellular domain of the receptor to create a new amino terminus, which can then act as a tethered ligand to activate the receptor. Peptides as short as the first 5 amino acids derived from the sequence of the proposed new amino terminus are able to fully activate human platelets and naive cells transfected with the clone for the thrombin receptor (5–8). Receptor activation on platelets results in the functional responses of platelet aggregation, secretion, Ca^{++} mobilization, protein phosphorylation, phosphatidyl inositol metabolism and inhibition of adenylate cyclase activity (9, 10). Thus, the receptor peptides can mimic many of the actions of thrombin itself in platelets.

Evidence for the existence of the cloned thrombin receptor has been presented for a wide variety of cells including endothelial cells, smooth muscle cells, mesenchymal-appearing cells and macrophages (reviewed in 4). In order to further explore the universality of this receptor and its mode of activation and role in normal hemostasis and thrombosis, we examined the effect of human thrombin receptor-derived peptides on the activation of platelets and other cells from a variety of animal species. These studies revealed that platelets from many species are not fully responsive to these receptor-derived peptides, despite being responsive to thrombin. However, other cells from these same species do give a full physiological response to the peptides.

Methods

Materials

Equine tendon type I collagen and ADP were from Chrono-Log Corp., Havertown, PA.; thrombin for the vascular reactivity studies was from Sigma, for the tissue culture studies it was from American Diagnostica, and for all other studies it was kindly provided by Dr. John W. Fenton II, Albany, N. Y.; human fibrinogen was from Calbiochem; CCL39 cells were obtained from the ATCC (Rockville, MD); peptides were from ABI; all other reagents were from Fisher and Sigma.

Platelet Preparation

Blood was collected from non-human species, or from healthy human volunteers free of aspirin and other drugs for at least 8 days, into 3.8% trisodium

citrate anticoagulant (1:10). The platelet rich plasma (PRP) was prepared by centrifugation and the platelet count was adjusted to 3×10^8 per ml with autologous platelet poor plasma. This PRP was used for all aggregations of platelets from the various animal species. For some studies human platelets were isolated by differential centrifugation and then washed in a modified Tyrode's buffer (5 mM HEPES, 0.3 MM NaH_2PO_4, 3 mM KCl, 134 mM NaCl, 5 mM glucose, 2 mM $MgCl_2$, 12 mM $NaHCO_3$, pH 6.5), containing 1 mM EGTA, 20 μg/ml apyrase and 3.5 mg/ml BSA as previously described (11). The final platelet suspension was at 2×10^8 platelets per ml in the same buffer at pH 7.4, without EGTA and apyrase.

Platelet Shape Change and Aggregation Assay

PRP or the washed platelets with 0.2 mg/ml human fibrinogen were incubated for 2 min at 37° C. Thrombin or a thrombin receptor-derived peptide was added to the sample in a Chronolog aggregometer and shape change and aggregation was monitored as a decrease in light transmittance, respectively. When thrombin was used as an agonist the PRP was pre-incubated for 2 min with 2 mM Gly-Pro-Arg-Pro peptide in order to prevent thrombin-mediated fibrin polymerization (12). The final amount of light transmittance (extent of aggregation) or the rate of change of light transmittance (rate of aggregation) was calculated by the aggrolink software provided by Chronolog Corp. with the aggregometer. In all cases qualitatively comparable results were obtained when either the rate or extent of aggregation was monitored.

Peptide Synthesis

Thrombin receptor peptides were synthesized as previously described (13). Briefly, peptide resins were assembled on solid support using an Applied Biosystems model 430A automated peptide synthesizer (t-BOC based chemistry). Peptides as carboxyl terminal amides (indicated by the symbol-NH_2) were synthesized on the benzylhydrylamine resin hydrochloride. The protected peptide-resins were treated with anhydrous liquid HF containing 10% anisole for 60 min at 0° C. The crude peptide products were purified by preparatory HPLC on a DELTA-PAK C_{18} column. Fractions containing product of at least 99% purity were combined and characterized by NMR and for amino acid composition after 6N HCl acid hydrolysis.

Cell Proliferation Assays

Cell culture of CCL39 cells and thymidine incorporation were carried out as previously described (13). Briefly, the cells were seeded in DME-10% FCS at 50,000 cells/well in cluster 24 plates (1 ml/well), grown to confluence, washed with phosphate buffered saline and exchanged into DME : Ham's F-12 (1:1) for growth arrest for 28 h. At this time the cells were exchanged into the same medium containing 1μCi/ml [3H]-methyl thymidine, 1 μg/ml cold thymidine and either 10% FCS, thrombin or thrombin receptor peptide. After 22 h the cells were washed with ice cold phosphate buffered saline, and treated with 1 ml 10% TCA for 10 min followed by rinsing with ethanol : ether (2 : 1), drying, solubilization and monitoring for radioactivity. Background, defined as the counts measured with exposure to medium only, was typically <8% that observed with FCS and was subtracted from all samples. Results are expressed as % thymidine incorporation relative to the FCS control in each experiment. All samples were tested in duplicate.

Vascular Smooth Muscle Tension Generation

Male purpose-bred mongrel dogs (9.2–10.3 kg) were anesthetized with sodium pentobarbital (35 mg/kg, i.v.) and mechanically ventilated with room air (Harvard Apparatus). A left thoracotomy was performed and the heart was excised and immersed in oxygenated, warmed (37° C) physiological salt solution (PSS: 112 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 1.0 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.6 mM dextrose and 1.1 μM ascorbic acid). The buffer was equilibrated at 37° C with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. The left circumflex artery was dissected free from the heart, blood was rinsed from the lumen, connective tissue and fat were carefully removed and the vessel was sectioned into rings of 2 mm in length. For some of the experiments, the endothelium was removed by inserting a 23 gauge needle adapter into the ring and rolling it gently on a moistened paper towel. To record force generation, each ring was placed between two stainless-steel wires within individual tissue bath chambers containing 15 ml of 37° C oxygenated PSS. The lower wire was immobilized while the upper wire was connected to a Statham UC2 tranducer. Each ring, with or without endothelium, was maintained at a baseline force of 1 gram during an equilibration period of 60 min. Tissues were primed with 40 mM KCl (2 times), following the wash-out after each KCl stimulation, baseline force was re-established at 1 gram. Arterial rings were then evaluated for their response from baseline to 1.0 μM of the thrombin receptor-derived activating peptide, SFLLRN-NH_2, or for the effect of 100 nM thrombin on vessels precontracted with 3 nM endothelin. Adequate de-endothelialization was confirmed by the addition of acetylcholine (0.1 μM) to the contracted preparations. In separate experiments, the response to cumulative doses of the peptide was demonstrated. Isometric tension data were collected on line at 2 s intervals and stored on a Compaq (Proline 4/33) computer system with software provided by Branch Technology. Digital data and a real time tracing were continuously displayed on a Flex Vision color monitor (model PMV/1448) with a hard copy provided by a Hewlett Packard laser jet PCL5. Hard copy analog data were continously recorded on a 8-channel Hewlett Packard 7758A.

Results

Aggregation of Platelets from Various Species in Response to Thrombin Receptor-derived Peptides

A systematic evaluation of platelet aggregation provoked by 5-residue (SFLLR-NH_2) or 14-residue (SFLLRNPNDKYEPF) peptides derived from the sequence of the human thrombin receptor was undertaken in platelet rich plasma from a variety of different animal species. As shown in Table 1, of the 9 different species evaluated only platelets from primates and those from the guinea pig demonstrated a full aggregation response. Platelets from a variety of species commonly used in thrombosis studies, including dog, hamster, rabbit, and pig showed a shape change but failed to aggregate in response to up to 800 μM peptide. Representative responses are shown in Figs. 5 and 7 as discussed below. Rat platelets did not aggregate and also did not change shape in response to the peptides. In contrast, only 1–10 μM of these peptides were required to promote full aggregation of human platelets. Receptor-derived peptides of intermediate length also failed to induce

Table 1 Effect of peptides derived from the human thrombin receptor on the aggregation of platelets from various animal species

Species	Aggregation[a]	Shape change
Human	87 ± 5	YES
Baboon	70 ± 4	YES
African green monkey	57 ± 9	YES
Rhesus monkey	68 ± 12	YES
Rabbit	3 ± 1	YES
Dog	0	YES
Pig	14 ± 2	YES
Rat	0	NO
Hamster	0	YES
Guinea pig	65 ± 13	YES

[a] Platelet rich plasma was challenged by up to 800 μM SFLLRN-NH_2, SFLLRN-NH_2 or SFLLRNPNDKYEPF peptides and aggregation and shape change monitored as described in Methods. Aggregation values are expressed as the maximal extent of aggregation observed ± the standard deviation. Results are from 3–6 separate experiments for each species.

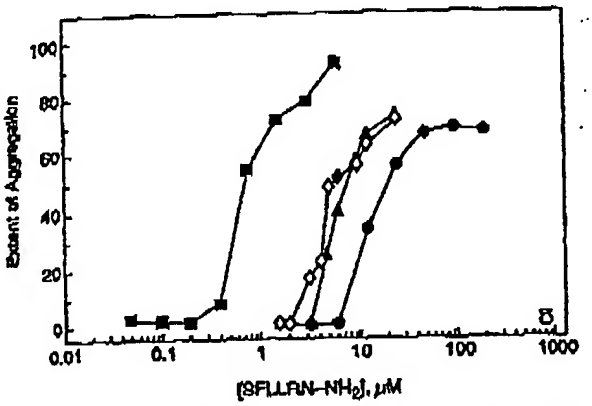


Fig. 1 Human thrombin receptor peptide stimulated platelet aggregation. PRP from the indicated species were challenged with the human thrombin receptor-derived peptide, SFLLRN-NH_2, and aggregation monitored as described in Methods. These results are the average of 2 separate experiments for each species, human (■), guinea pig (▲), Rhesus monkey (◇), baboon (●), dog (▽) and hamster (○)

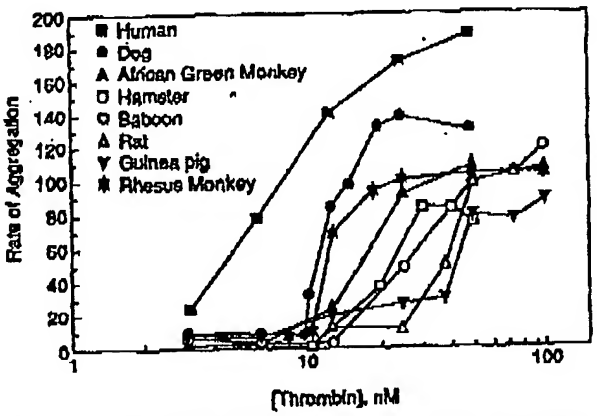


Fig. 2 Thrombin-stimulated platelet aggregation. PRP (0.25 ml) from the indicated species was prepared and stimulated by the indicated concentrations of human thrombin and aggregation monitored as described in Methods. These results are the mean rate of aggregation for human (■), n = 4; dog (●), n = 2; African green monkey (▲), n = 2; hamster (□), n = 1; baboon (○), n = 2; rat (△), n = 3; guinea pig (▼), n = 2; and Rhesus monkey (*), n = 2

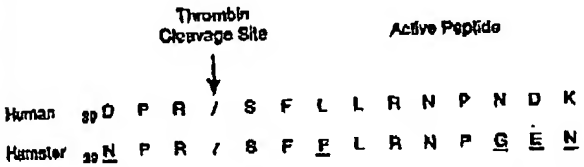


Fig. 3 Differences in the thrombin receptor sequence in various species. The third residue of the activating peptide from the hamster (3), mouse (14), and rat (15) has an F (Phe) substitution for L (Leu). Underlined residues indicate differences from the human sequence (2)

Table 2 Platelet aggregation of non-human, hamster, pig and rat, platelets in plasma or human washed platelets was carried out as described in Methods. The results are from at least 3 separate experiments for each peptide in each species

Peptide	Aggregation EC_{50}, μM	
	Non-human platelets	Human platelets
SFLLR-NH_2	>800	1
SFLAR-NH_2	>800	15
SFALR-NH_2	>800	1.3
A(3Qual)LLR-NH_2*	>800	>800
A(hF)LLR-NH_2*	>800	>800

* 3Qual = 3-(3quinolyl)alanine, hF = homo phenylalanine.

aggregation (data not shown). Platelets from the responding species of guinea pigs, Rhesus monkeys and baboons, however, fully aggregated in response to the human receptor peptides, although 10 to 40-fold more peptide than that needed to promote aggregation of human platelets was required for an equivalent response as shown in Fig. 1.

The platelets from the species which did not aggregate in response to human thrombin receptor-derived activating peptides, did aggregate after stimulation by human thrombin as shown in Fig. 2, and by ADP and collagen, two other platelet agonists which act via distinct receptors (not shown). This result demonstrates that the platelets from these species can aggregate and that they have thrombin receptors that are recognized and activated by human thrombin.

A comparison of the sequences of the thrombin receptors deduced from cDNA clones of the thrombin receptor from human (2), hamster (3), mouse (14) and rat (15) sources is shown in Fig. 3. In the proposed activating peptide region there is a substitution of a Phe for Leu at position 44 in the rodent species. We speculated that activating peptides with this substitution might activate platelets from homologous species, that had not responded to the human receptor-derived peptides. A 7-residue peptide incorporating this substitution was prepared and its effect on the aggregation of human platelets compared to that elicited by the

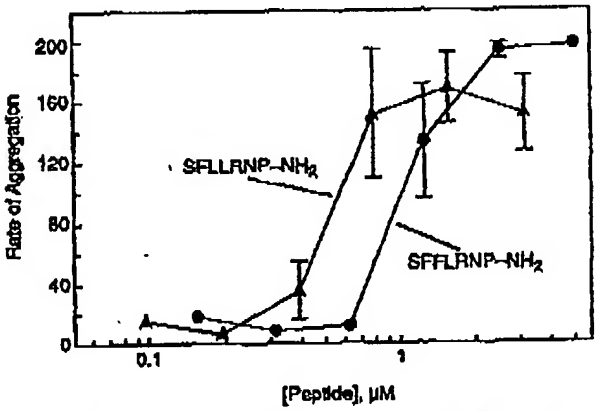


Fig. 4 Peptide-stimulated human platelet aggregation. Washed human platelets were stimulated by 7-residue peptides based on the sequence of the human thrombin receptor, SFLLRNP-NH_2 (▲), or the hamster thrombin receptor, SFFLRNP-NH_2 (●), and aggregation monitored as described in Methods. The results are expressed as mean ± S. E. for 3 separate experiments for each peptide

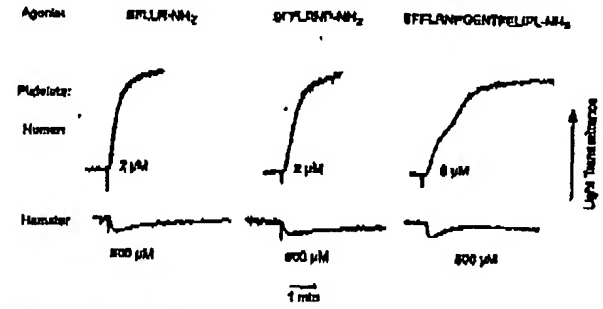


Fig. 5 Aggregation of human and hamster platelets. Human or hamster PRP was stimulated by the indicated concentration of human thrombin receptor-derived peptide, SFLLR-NH_2, or the hamster thrombin receptor-derived peptides, SFFLRNP-NH_2 and SFFLRNPGENTFELIPL-NH_2, and aggregation monitored as described in Methods and as shown by the tracings. These results are representative of 3–5 separate experiments

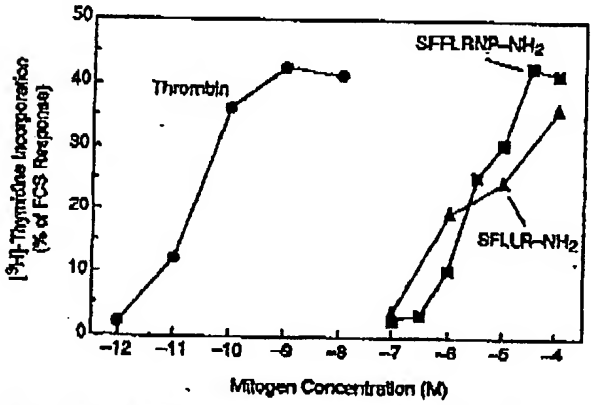


Fig. 6 Mitogenic activity of thrombin receptor agonist peptides towards CCL39 cells. (●) human thrombin, (▲) a human thrombin receptor-derived peptide, SFLLR-NH_2, or (■) the hamster thrombin receptor-derived peptide, SFFLRNP-NH_2, were added to confluent cultures of growth arrested CCL39 cells and after 22 H the extent of DNA synthesis was determined by measuring [3H]-Thymidine incorporation into newly synthesized DNA as described in Methods and (13). The data are expressed as [3H]-Thymidine incorporation as a percentage of the response to 10% fetal calf serum (FCS) in the same experiment

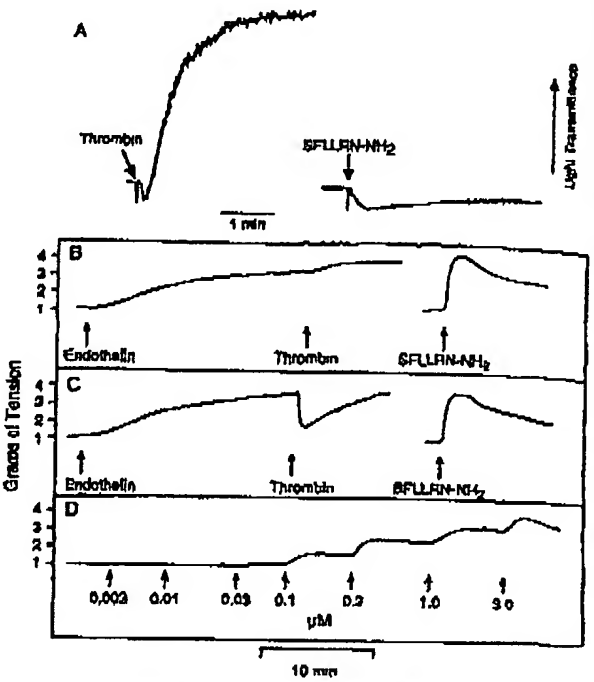


Fig. 7 Effect of thrombin and a thrombin receptor peptide on canine tissues. (A) Aggregation of dog PRP to 25 nM thrombin or 800 μM SFLLRN-NH_2 peptide was carried out as described in Methods. Vascular response of canine coronary artery rings without (B) and with endothelium (C, D) to 100 nM thrombin or 1 μM SFLLRN-NH_2 peptide carried out as described in Methods. (D) Dose-related response of canine coronary vessel rings to SFLLRN-NH_2 peptide added cumulatively. Tracings of B-D are representative of 4–5 separate experiments

corresponding 7-residue human receptor peptide. As shown in Fig. 4 the rodent-derived receptor peptide also promoted full aggregation of human platelets similarly to that induced by the human receptor-derived peptide.

The effect of the hamster receptor-derived peptide together with that of the human receptor-derived peptide of the same length on hamster platelet aggregation is illustrated in Fig. 5. The human platelets in plasma showed comparable aggregation in response to 2 μM of each peptide, however, both the human- and rodent-derived peptides at up to 800 μM failed to promote aggregation of hamster platelets despite the latter being from the same species. In order to rule out the possibility that a 7 residue hamster receptor peptide is of insufficient length to activate the hamster platelets, a longer peptide of 17 amino acids derived from the cleavage site of the hamster receptor clone was prepared. This peptide at up to 800 μM also did not induce aggregation of the hamster platelets despite being a potent activator of human platelet aggregation as shown in Fig. 5. Stimulation of rodent and pig platelets by 5-residue peptides with an alanine substitution in position 3 or substitutions in the 2 and 4 positions with other residues in an attempt to mimic their endogenous receptor ligands also did not result in peptides which could initiate aggregation of these platelets as shown in Table 2.

In order to determine if peptides were being inactivated by some plasma component both human receptor-derived activating peptides of 5 and 14 amino acids and the 7 and 17 amino acid rodent-derived peptides were incubated with plasma from nonaggregating species for 5 min. at 37° C and then added to human platelets at 2 times their EC_{50} concentration to determine if the plasma from these species was inactivating the peptides. These platelets aggregated with a response comparable to that observed in response to the same concentration of peptide not pre-incubated with the plasma. Thus, there was no peptide inactivation in the plasma from these non-responding species under these conditions.

Mitogenic Response of CCL39 Cells

We had previously observed that thrombin and human thrombin receptor peptides could promote increased incorporation of [3H]-thymidine in CCL39 cells, a hamster-derived fibroblast cell line (13). Since hamster platelets did not aggregate in response to either human or hamster thrombin receptor-derived peptides it was of interest whether

or not hamster fibroblasts fully respond to the hamster-derived peptides. As shown in Fig. 6 and as previously reported (13), thrombin and both human and hamster-derived receptor peptides induced a strong mitogenic response in CCL39 cells. Although the potencies of each to elicit these responses was different, the same final extent of [^{3}H]-thymidine was incorporated in response to each stimulus.

Effect of Thrombin and a Thrombin Receptor Peptide on Canine Vascular Response

We next explored whether the apparent tissue selective responsiveness to thrombin receptor-derived peptides was unique to the hamster or if thrombin responsive tissues in other species could respond to these peptides. Dog platelets did not aggregate in response to human thrombin receptor-derived peptides (Fig. 1 & 7A) but they did aggregate to thrombin stimulation (Fig. 2). The effect of thrombin and the human thrombin receptor-derived peptide, SFLLRN-NH_2, on isolated canine coronary artery rings was evaluated. Thrombin induced a moderate contraction (22.4% or 0.48 g increase) of 3 nM endothelin precontracted (2.14 g) de-endothelialized rings (Fig. 7B), and elicited a marked relaxation (72.1% or 1.78 g decrease) of endothelin precontracted (2.47 g) coronary artery rings with endothelium from the same vessel (Fig. 7 C). The endothelin-stimulated contraction (3 g active tension) was similar to that typically elicited by 40 mM KCl (85% of the KCl response in the denuded rings and 65% of the KCl response in the intact vessel segments). In contrast to its effect on canine platelets, the 6-residue thrombin receptor peptide elicited a dose-related contraction above baseline tension levels in the canine coronary artery rings both without (Fig. 7 B) and with endothelium (Fig. 7 C & D). The magnitude of these contractions were 2.95 g or 95% of the response to 40 mM KCl without endothelium and 2.76 g or 75% of the response to KCl in the same vessel with intact endothelium.

Discussion

Thrombin plays a vital role in many aspects of normal hemostasis (reviewed in 16). It is a procoagulant enzyme, mediating the conversion of fibrinogen to fibrin and the activation of Factors V and VIII, thereby promoting its own production. Thrombin can also act as a feedback inhibitor of its own production by activating the natural anticoagulant, protein C (17). It also directly activates platelets and many other cell types via a cell surface receptor(s) (1). With the cloning, expression and elucidation of the mechanism of activation of a human thrombin receptor on megakaryocytic-like cells (4) it became possible to directly examine activation of this thrombin receptor on platelets and other cells without interference from the other responses to thrombin.

According to the proposed mechanism of activation of this receptor (4) peptides derived from the newly created amino terminus should activate platelets directly via this receptor. In the current studies we examined the ability of peptides derived from the amino acid sequence deduced from the clone of the human or rodent thrombin receptor to promote aggregation of platelets from a variety of different species. We found that in addition to human platelets only those from primates and the guinea pig (Table 1) fully aggregated in response to these peptides. This result is in agreement with the findings of Kinlough-Rathbone et al. (18) and Catalfamo et al. (19). The non-aggregating platelets from the dog, rat, rabbit and hamster, however, were physiologically competent as they did aggregate in response to other agonists such as ADP or collagen. They also have a thrombin receptor, as they aggregated in response to human thrombin (Fig. 2). The platelets are partially activated by the peptides derived from the thrombin receptor, as the platelets from all species tested (except the rat) underwent a shape change in response to these peptides.

The precise mechanistic basis for the inability of the peptides with sequences derived from the human thrombin receptor to promote full aggregation of platelets from the non-primate species is unknown. It is unlikely that the inability of the human receptor peptides to promote full aggregation of platelets from many species is due simply to a lower affinity of the same thrombin receptor for these peptides (all of which fully respond to thrombin). Kinlough-Rathbone et al. (18) tested the effect of 100 μM of the thrombin receptor peptide with the animal species, a 20-fold increase over the amount required to promote full aggregation of human platelets. In the current studies an 800-fold excess of peptide over that which induced full aggregation of human platelets in plasma was unable to elicit aggregation of platelets from non-responding species. The lack of aggregation in response to a concentration of peptide almost three orders of magnitude greater than that required to promote full aggregation of human platelets suggests that the receptors are pharmacologically distinct between species. Likewise, because shape change and not aggregation was provoked by the receptor peptides in several non-primate species, pharmacologically distinct thrombin receptors within these species receptors may mediate these responses.

An alternative explanation for the lack of aggregation of platelets from many species could be explained by a high amount of aminopeptidase M activity in plasma from the non-responding species which would cleave the amino terminus serine from the activating peptides, thereby rendering them inactive as described by Coller et al. (20). This is however, unlikely, because incubation of the peptides with platelet rich plasma from the various non-responding species did not render them incapable of promoting aggregation of human platelets (not shown). In addition, at the peptide concentration examined (800 μM) the peptidase activity would have to hydrolyze more than 99.9% of the peptide within a few seconds.

The lack of aggregation response of platelets from some species to the receptor peptides is unlikely due to a difference in thrombin receptors that is unique to the non-responding species or to platelets within a given species. It could be argued that the platelets from the non-responding species do not aggregate because the sequence of their thrombin receptor in the region of the tethered ligand is different at some critical residue, and therefore, their activating peptide would be different than that of the human-derived peptide. Structure activity studies (5-8, 21) indicate that the second residue of the activating against peptides is the most critical for agonist activity. Several peptides at up to 800 μM with substitutions in this residue, and in residues 3 and 4 did not promote platelet aggregation of the non-responding species, while being effective agonists for human platelet aggregation (Table 2). Cloning of the thrombin receptor from rodents, including hamster (3), mouse (14), and rat (15), revealed a substitution of a Phe for Leu at the third residue. Peptides of 5 to 14 residues in length derived from the hamster receptor sequence stimulated the aggregation of human platelets, but the effect of this peptide on platelets from other species, including hamster, was not previously reported (22). We found that a 7 or 17 residue hamster receptor peptide was nearly as effective as the human sequence to promote aggregation of human platelets, but at up to 800 μM did not provoke aggregation of platelets from hamster the species from which these peptides were derived (see Fig. 3). The lack of a response of the hamster platelets to the hamster-derived sequence is inconsistent with this simple explanation and suggests a more complex mechanism of receptor activation.

In the current studies, peptides derived from the human thrombin receptor could not promote aggregation of hamster platelets, yet previous studies had demonstrated that these peptides could either promote phosphatidyl inositol turnover and inhibition of adenylate cyclase (22) or mitogenesis (13, 23) in CCL 39 cells, a hamster fibroblast cell line. The current studies confirm the ability of human peptides to promote mitogenesis in these cells and demonstrate that a hamster-derived peptide also promotes mitogenesis of these hamster fibroblast cells. Also, we found that human receptor peptides stimulated smooth muscle contraction in coronary artery rings from dogs, despite not being able to induce aggregation of their platelets. Previous work had demonstrated endothelium-dependent relaxation of canine saphenous veins and contraction of dog coronary artery strips by human thrombin receptor peptides (24). Rat platelets also did not aggregate in response to the human receptor peptides. Kinlough-Rathbone et al. (18) and Catalfamo et al. (19) also reported a similar finding. Yet, despite the lack of any rat platelet functional response to 800 μM human or rodent receptor peptide, human peptides have been shown to have an effect on vascular reactivity of rat tissues (25–27) and to be mitogenic for rat vascular smooth muscle cells in culture (28). Therefore, in at least these three species the thrombin receptors on non-platelet cells have different pharmacological properties than those on their platelets.

Although the molecular basis of this pharmacological difference in receptors is not yet known, these results suggest two possibilities for further exploration. First, there may be more than one thrombin receptor as has been previously suggested (reviewed in 29); the cloned receptor on primate and hamster platelets, which responds to both thrombin and receptor-derived peptides, and another receptor(s) on platelets from non-primate species that is molecularly unrelated and only fully activated by thrombin. The currently available molecular biological approaches would identify only receptors structurally related to the cloned receptor. Data from several other types of approaches do support the existence of more than one thrombin receptor. First, platelet activation induced only by lower concentrations of thrombin is antagonized by antibodies against the exosite region of the cloned thrombin receptor (30-32) and by PPACK-thrombin (33). Higher concentrations of thrombin can overcome this inhibition and thus may interact with a distinct receptor. Secondly, Seiler et al. (34) reported on two types of responses to thrombin in human platelets and suggested the existence of more than one receptor. One response was to low concentrations of alpha thrombin but not gamma thrombin and it was desensitized by receptor-derived peptides, while the other was to higher concentrations of alpha thrombin and to gamma thrombin and was not desensitized by the receptor peptides. A variety of thrombin binding proteins have been identified on platelet surfaces and have been implicated as being important for thrombin to promote activation in platelets and other cell types (35). Indeed, thrombin has been shown to bind to human platelets with at least three different affinities (36). The second possibility is that the receptor peptides may be partial agonists of the platelets from the non-aggregating species. In most cases the human and hamster-derived peptides could evoke a shape change response of the platelets. This suggests that a receptor interaction that initiates intracellular signaling sufficient to promote shape change but not aggregation in these species is occurring. Indeed, only the platelets from the rat showed no response to the peptides and pig platelets actually aggregated ~13% of full aggregation. Kinlough-Rathbone et al. reported that pig platelets fully aggregated in response to human peptides but those from rabbit and rat did not (18). The receptor which responds to the peptides may couple to different effector systems in the non-responding cells that do not allow, for example, the expression of competent fibrinogen receptors or some other requirement for aggregation. The further comparison of the effects of these peptides on platelets from primate and non-primates may allow for the distinction between intracellular events that elicit only shape change and those that elicit shape change and aggregation as induced by thrombin.

Acknowledgements

We would like to thank Dr. Joseph Lynch, Dr. Linda Schaffer and Tsuneo Fujita for their assistance with the animal species, Ernest Mayer for assistance with the mitogenesis studies, Monica Norris for technical contributions in the smooth muscle experiments and Drs. Bohumil Bednar and Jerzy Karczewski for helpful discussions.

References

1. Shuman MA. Thrombin-cellular interaction. In: Annals of N. Y. Acad. Sci. Vol 485: Awtz DA, Fenton II JW, Shuman MA, eds. New York Academy of Sciences, New York, NY 1986: pp 228–39.
2. Vu T-KH, Hung DT, Wheaton VI, Coughlin SR. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor activation. Cell 1991; 64: 1057–68.
3. Rasmussen UB, Vouret-Craviari V, Jallat S, Schlesinger Y, Pages G, Pavirani A, Lecocq J-P, Pouysségur J, Van Obberghen-Schilling E. cDNA cloning and expression of a hamster-thrombin receptor coupled to Ca^{2+} mobilization. FEBS 1991; 288: 123–8.
4. Coughlin SR, Vu T-KH, Hung DT, Wheaton VI. Characterization of a functional thrombin receptor, issues and opportunities. J Clin Invest 1992: 89: 351–5.
5. Scarborough RM, Naughton MA, Teng W, Hung DT, Rose J, Vu T-KH, Wheaton VI, Turck CW, Coughlin SR. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal a mechanism of proteolytic unmasking of agonist function. J Biol Chem 1992: 267: 13146–9.
6. Vassallo Jr RR, Kieber-Emmons T, Chichowski K, Brass LF. Structure-function relationships in the activation of platelet thrombin receptors by receptor derived peptides. J Biol Chem 1992; 267: 6081–5.
7. Chao BH, Kalkunte S, Maraganore JM, Stone SR. Essential groups in synthetic agonist peptides for activation of the platelet thrombin receptor. Biochem 1992; 31: 6175–8.
8. Hui KY, Jakubowski JA, Wyss VL, Angleton EL. Minimal sequence requirement of thrombin receptor agonist peptide. Biochem Biophys Res Comm 1992; 184: 790–6.
9. Seiler SM, Michel IM, Fenton II JW. Involvement of the tethered ligand receptor in thrombin inhibition of platelet adenylate cyclase. Biochem Biophys Res Comm 1992; 182: 1296–1302.
10. Hung R-S, Sorisky A, Church WR, Simons ER, Rittenhouse SE. Thrombin receptor-directed ligand accounts for activation by thrombin of platelet phospholipase C and accumulation of 3-phosphorylated phosphoinositides. J Biol Chem 1991; 266: 18435–8.
11. Connolly TM, Jacobs JW, Condra C. An inhibitor of collagen-stimulated platelet activation from the salivary glands of the Haementeria officinalis leech. J Biol Chem 1992: 267: 6893–8.
12. Plow EF, Marguerie G. Inhibition of fibrinogen binding to human platelets by the tetrapeptide glycyl-L-prolyl-L-Arginyl-L-proline. Proc Natl Acad Sci 1982; 79: 3711–5.
13. Reilly CF, Connolly TM, Feng D-M, Nutt RF, Mayer EJ. Thrombin receptor agonist peptide induction of mitogenesis in CCL 39 cells. Biochem Biophys Res Comm 1993: 190: 1001–8.
14. Vu T-KH, Wheaton VI, Hung DT, Charo I, Coughlin SR. Domains specifying thrombin-receptor interaction. Nature 1991: 353: 674–7.
15. Zhong C, Hayzer DJ, Corson MA, Runge MS. Molecular cloning of the rat vascular smooth muscle thrombin receptor: evidence for in vitro regulation by basic fibroblast growth factor. J Biol Chem 1992: 267: 16975–9.

16. Fenton JW II. Regulation of thrombin generation and functions. Sem in Thromb and Hemost 1988; 14: 234–40.
17. Esmon CT. The regulation of natural anticoagulant pathways. Science 1987; 235: 1348–52.
18. Kinlough-Rathbone RL, Rand ML, Packham MA. Rabbit and rat platelets do not respond to thrombin receptor peptides that activate human platelets. Blood 1993; 82: 103–6.
19. Catalfamo JL, Anderson TT, Fenton II JW. Thrombin receptor activating peptides unlike thrombin are insufficient for platelet activation in most species. Thromb Haemost 1993; 69: 1195 (abstract).
20. Coller BS, Ward P, Ceruso M, Scudder LE, Springer K, Kutok J, Prestwich GD. Thrombin receptor activating peptides: importance of the N-terminal serine and its ionization state as judged by pH dependence, nuclear magnetic resonance spectroscopy, and cleavage by aminopeptidase M. Biochem 1992; 31: 11713–20.
21. Connolly TM, Condra C, Feag D-M, Nutt RF, Gould RJ. Activation of human platelets by potent thrombin receptor-derived peptides. Thromb Haemost 1993; 69: 787 (abstract).
22. Vouret-Craviari V, Van Obberghen-Schilling E, Rasmussen UB, Pavirani A, Lecocq J-P, Pouysségur J. Synthetic-thrombin receptor peptides activate G protein-coupled signaling pathways but are unable to induce mitogenesis. Mol Biol of Cell 1992; 3: 95–102.
23. Hung DT, Vu T-HK, Nelken NA, Coughlin SR. Thrombin-induced events in non-platelets cells are mediated by the unique proteolytic mechanism established for the cloned platelet thrombin receptor. J Cell Biol 1992; 116: 827–32.
24. Simonet S, Bonhomme E, Laubie M, Thurleau C, Fauchère J-L, Verbeuren TJ. Venous and arterial endothelial cells respond differently to thrombin and its endogenous receptor agonist. Eur J Pharmacol 1992; 216: 135–7.
25. Hollenberg MD, Yang S-G, Laniyonu AA, Moore GJ, Saifeddine M. Action of a thrombin receptor polypeptide in gastric smooth muscle: identification of a core pentapeptide retaining full thrombin-mimetic activity. Mol Pharmacol 1992; 42: 186–91.
26. Muramatsu I, Laniyonu AA, Moore GJ, Hollenberg MD. Vascular actions of thrombin receptor peptide. Can J Physiol Pharmacol 1992; 70: 996–1003.
27. Antonaccio MJ, Normandin D, Serafino R, Moreland S. Effects of thrombin and thrombin receptor activating peptides on rat aortic vascular smooth muscle. J Pharmacol Exp Ther 1993; 266: 125–32.
28. McNamara CA, Sarembock IJ, Gimple LW, Fenton II JW, Coughlin SR, Owens GK. Thrombin stimulates proliferation of cultured rat aortic smooth muscle cells by a proteolytically activated receptor. J Clin Invest 1993; 91: 94–8.
29. Simons ER, Davies TA, Greenberg SM, Larsen NE. Thrombin receptors on human platelets. In: Methods Enzymol. Hawiger J, ed. Academic Press, San Diego, CA 1992; 215: 155–76.
30. Hung DT, Vu T-KH, Wheaton VI, Coughlin SR. Cloned platelet receptor is necessary for thrombin-induced platelet activation. J Clin Invest 1992; 89: 1350–3.
31. Brass LF, Vassallo Jr RR, Belmonte E, Ahuja M, Cichowski K, Hoxie JA. Structure and function of the human platelet thrombin receptor. Studies using monoclonal antibodies directed against a defined domain within the receptor N terminus. J Biol Chem 1992; 267: 13795–8.
32. Bahou WF, Coller BS, Potter CL, Norton KJ, Kutok J, Goligorsky MS. The thrombin receptor extracellular domain contains sites crucial for peptide ligand-induced activation. J Clin Invest 1993; 91: 1405–13.
33. Greco NJ, Tenner Jr TE, Tandon NN, Jamieson GA. FPACK-thrombin inhibits thrombin-induced platelet aggregation and cytoplasmic acidification but does not inhibit platelet shape change. Blood 1990; 75: 1983–90.
34. Seiler SM, Goldenberg HJ, Michel IM, Hunt JT, Zavoico GB. Multiple pathways of thrombin-induced platelet activation differentiated by desensitization and a thrombin exosite inhibitor. Biochem Biophys Res Comm 1991; 181: 636–43.
35. Takamatsu J, Horne III MK, Gralnick HR. Identification of the thrombin receptor on human platelets by chemical crosslinking. J Clin Invest 1986; 77: 362–8.
36. Harmon JT, Jamieson GA. Thrombin binds to a high affinity ~900000-dalton site on human platelets. Biochem 1985; 24: 58–64.

Received April 29, 1994 Accepted after revision June 20, 1994